EXPERIMENTAL CELL RESEARCH

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VOLUME 6 · FEBRUARY 1954 · NUMBER 1

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

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Experimental Cell Research, **6**, *1–16 (1954)*

TORSION IN A PROTOPLASMIC THREAD[1]

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Received March 28, 1953

Spiral tendencies in biological systems are well known. They are revealed in structure, growth, and movement, at various levels of dimensions and organizations (5). Apparently no research has been done on spiralling in protoplasm except that mentioned in a brief report by Seifriz (6). He noticed that a state of torsion is established in a hanging strand of the slime mould, *Physarum polycephalum*.

The experiments described on the following pages demonstrate the twisting of a protoplasmic thread of a slime mould. The results obtained bear significantly on the dynamic functioning of protoplasm. The slime mould used was *Physarum polycephalum*, cultured by the method of feeding with oats (1).

EXPERIMENTAL

I. *Behaviour of a protoplasmic thread suspended in the air between two fixed ends*

An experiment is set up by taking a protoplasmic thread and two small masses (about 10 mg each) of protoplasm from the stock culture. The latter are placed on agar walls which face each other at a distance of 2 cm. The protoplasmic thread, which should be about 3 cm long with a diameter of 100–250 microns, is hung in the air between and in contact with the two protoplasmic masses on the agar walls. The two ends of the thread at first simply adhere to the protoplasmic masses, but the thread and two masses of protoplasm soon fuse into a single united plasmodium (Fig. 1). The endoplasm will then flow through the suspended thread with the same rhythmic reversal in direction of flow which is so typical of this slime mould. The slime mould is kept in a moist chamber to prevent the protoplasm from drying. This setup is the same as that developed by Kamiya and Abe (4) when they measured the electromotive force of the slime mould.

For a while after the above preparation is made the protoplasmic thread

[1] This work was supported by a grant from the National Cancer Institute, U.S. Public Health Service, and by a grant from the American Cancer Society upon recommendation of the Committee on Growth, for which we want to express our gratitude.

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hangs down in the form of a catenary, i.e. in the form represented by a chain suspended between two terminals, showing that the rigidity of the protoplasm is overcome by its own weight. On careful observation the protoplasmic thread can be seen to tighten and relax rhythmically with the same period as that of the protoplasmic flow. The thread can also be seen to pulsate through change in its diameter.

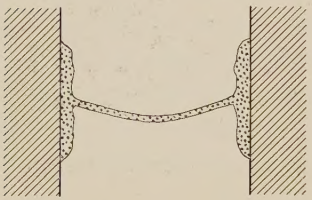

Fig. 1. A protoplasmic thread suspended in the air between two masses of protoplasm creeping on agar walls.

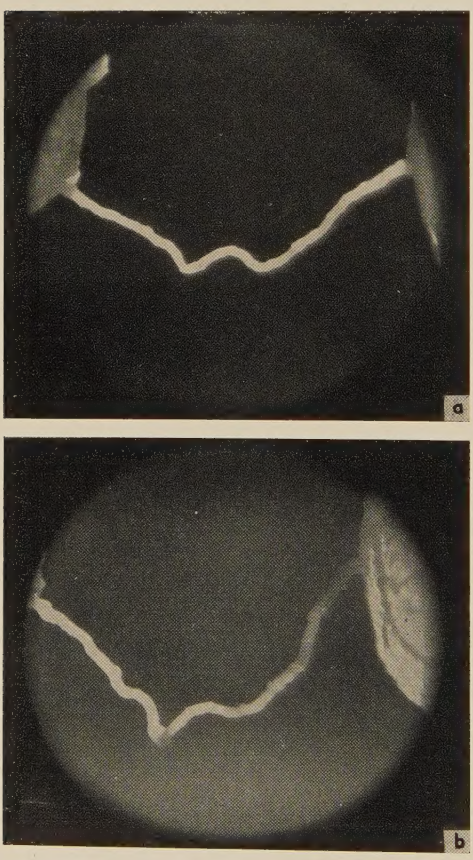

Fig. 2. *a:* The suspended thread of protoplasm showing a tendency to spiral. *b*: The same thread, several minutes later. Magnification: 3 ×.

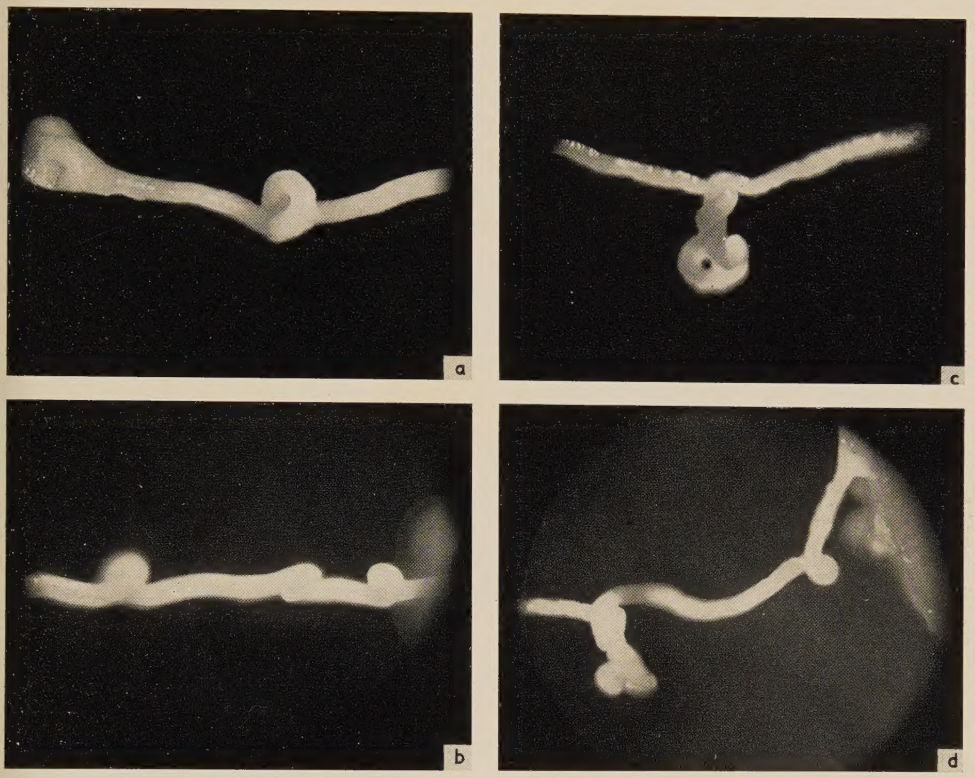

Fig. 3. Protoplasmic threads forming loops following the stages shown in Fig. 2. Magnification: 6 ×.

When the plasmodium is left under the foregoing conditions for a longer period of time, say 2 or 3 hours, it is noticed that the hanging thread gradually takes a form deviating from a catenary line. This form resembles that of a contorted rope. Figs. 2a and 2b show stages in which the protoplasmic thread is beginning to form a spiral. Soon after these changes occur, a portion of the thread is twisted into a loop just as is true of an inanimate thread when it is twisted. In Fig. 3 are shown some examples of protoplasmic threads forming loops. Fig. 3a represents a simple loop. If we refer to the manner of winding shown in Fig. 3a as right-handed, we see that in Fig. 3b there are two right-handed and one left-handed loops side by side in one and the same thread. In Fig. 3c the protoplasmic thread not only forms a loop but is twisted still further. This same thread, illustrated in Fig. 3c, formed another right-handed loop (Fig. 3d) about 10 minutes after the photograph in Fig. 3c was taken.

The formation of a spiral, or loops, in a thread of comparatively undifferentiated protoplasm is certainly a fascinating phenomenon. But if we want to make a quantitative analysis of the spiralling nature of protoplasm, the simple observation of the form of the thread is not sufficient.

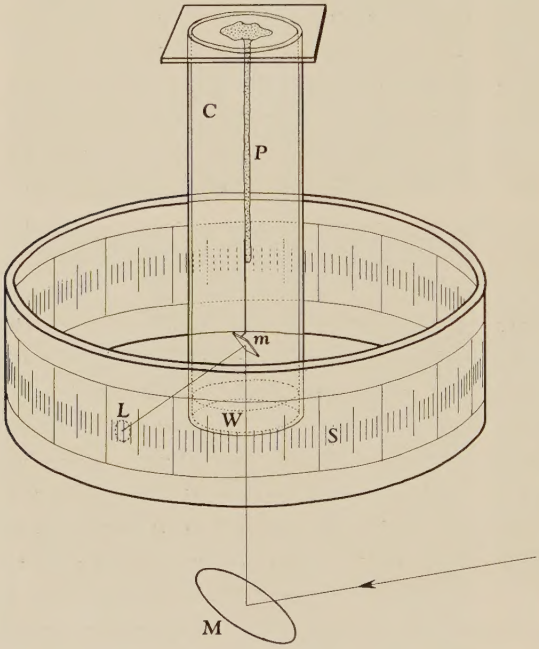

Fig. 4. General setup for measuring rotation of the lower end of the hanging protoplasmic thread. From the free end of the thread a tiny mirror is hung by means of a delicate rod. The mirror hangs at an angle of 45° with the horizontal. The thickness of the protoplasmic thread and the size of the mirror are drawn somewhat greater than the actual proportion. *S*: cylindrical scale; *P*: protoplasmic thread; *C*: moist chamber; *W*: water at the bottom of the moist chamber to prevent the material from drying; *m*: hanging, inclined mirror; *L*: light spot projected by the beam reflected at *m* onto the cylindrical scale; *M*: mirror to reflect the beam in a vertical direction.

II. *Behaviour of a protoplasmic thread suspended in the air by one end while the other end is left free*

The formation of a spiral or series of loops in a protoplasmic thread could be due to a torque somehow established in living matter. It was in the hope of obtaining further light on this question that we investigated the behaviour of a protoplasmic thread hung vertically in moist air. One end of the thread was fused with a protoplasmic mass creeping on the under surface of the lid of a moist chamber (*C*, Fig. 4) while the other, lower, end was left free. The purpose of the experiment was to determine how the tip of a hanging thread of protoplasm twists autonomously against its base (top) when it is free to rotate. For measuring the angle of rotation a tiny mirror (*m*, Fig. 4), measuring 1.5 × 3.0 mm, was provided. At its margin a delicate glass handle, 25–35 mm long, was fused at an angle of 45°. The

mirror, which, with its handle, weighed about 2 mg, was held by the protoplasmic thread in such a way that the lower portion of the thread and the upper portion of the handle of the mirror were attached parallel to each other by adhesion, the length of the adhering portion being 2–4 mm. (Fig. 4). The mirror rotates as the lower tip of the protoplasmic thread rotates, keeping its inclination at 45° against the horizontal plane. Thus, if a cylindrical scale (*S*) is placed around the moist chamber, and the protoplasmic thread with its mirror placed in the center, and if a beam of light is projected vertically from below (*M*), the mirror (*m*) will reflect the beam onto the cylindrical scale, as shown in Fig. 4. As the mirror rotates, together with the free end of the thread, the reflected beam also rotates. By reading the position of the bright spot projected on the scale at short intervals, it is not difficult to trace rather exactly the changes in the orientation angle of the reflected beam.

The net length of the hanging thread, i.e. the length excluding some stretch of the lower end where the handle of the mirror is attached, was mostly 2.0–6.0 mm in a thread 20–80 mm long. As to thickness of the thread, it varied both chronologically and spatially in one and the same specimen during the course of the experiment. Moreover, the thread often developed various kinds of protrusions, knots or tufts, all of which made it difficult for us to give a clear description of its thickness on a quantitative basis except to say that it changed usually within the range of 50–200 microns.

a) *Torsion.* In the first place it is to be noticed that the reflected beam swings to the right and left, or clockwise and counter-clockwise, as reported by Seifriz (6), usually with a period of about 2 minutes at room temperature. This means a repeated twisting and untwisting of the thread of living protoplasm. The angle of a single swing differs greatly with the specimen used and also with the time of measurement. It amounts usually to 1/2 revolution (180°), but it is not at all rare that a single stroke covers more than 1 revolution (360°), even 4 revolutions.

The orientation angle of the reflected beam was measured every 10 seconds, sometimes every 5, 15, or 20 seconds. By graphical representation of the time course of rotation of the lower end of the thread, the counter-clockwise rotation seen from above was designated as plus, and clockwise rotation as minus according to the custom in mathematics. By plotting the series of values representing the orientation of the reflected beam as ordinates against time as abscissas, the points plotted fall in an undulating curve which faithfully portrays the time course of rotation at the free end of the protoplasmic thread (Fig. 5).

The measurements used in Fig. 5 were made every 5 seconds. As the counter-clockwise direction of torsion has been designated as plus and the clockwise direction as minus in sign, the ascending portions of the undulating curve show the periods of a counter-clockwise rotation and the descending parts a clockwise rotation: the maxima and minima represent the moments of reversal. Here we notice that more or less similar waves are repeated in succession, but in each wave counter-clockwise rotation is predominant over

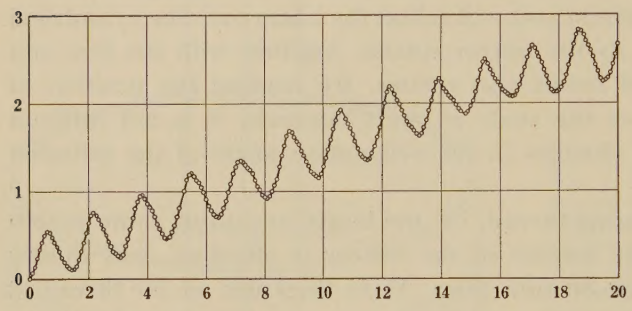

Fig. 5. Rhythmic reversal in rotation of the free end of the hanging protoplasmic thread. The undulating curve as a whole ascends gradually revealing dominance in the counter-clockwise rotation over the clockwise rotation. Length of thread: the 14 mm. Temp.: 24° C.

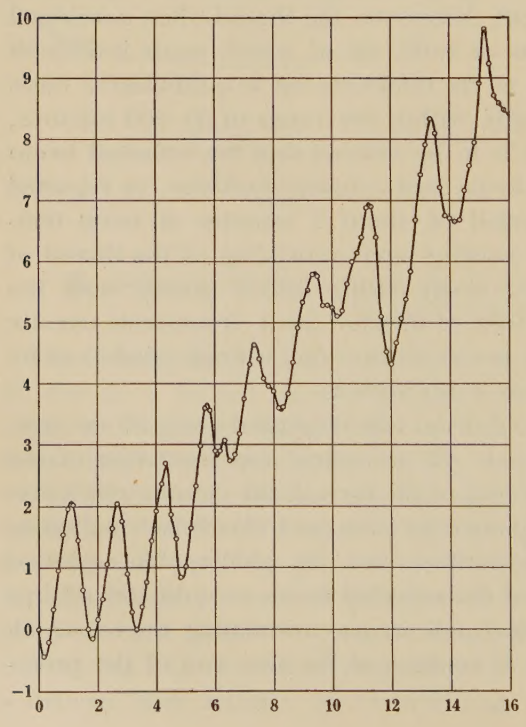

Fig. 6. Time course of rotation of the free end of the protoplasmic thread. The undulating curve shows a pronounced upward (counter-clockwise) trend. Length of the thread: 24 mm. Temp.: 26° C.

the clockwise rotation. A clockwise rotation reverses to the counter-clockwise rotation without coming all the way back to the initial state of orientation from which the preceding counter-clockwise rotation started. Repetition of this rhythmic motion naturally results in an increasingly greater twist of the protoplasmic thread in the counter-clockwise direction. This is true because the thread each time avails itself of the net difference between the twisting angles which it adds to the twist in one direction. This remarkable fact is indicated on the graph in Fig. 5 by the tendency of the undulating curve to ascend. From this figure we learn that the thread twisted itself 2.5

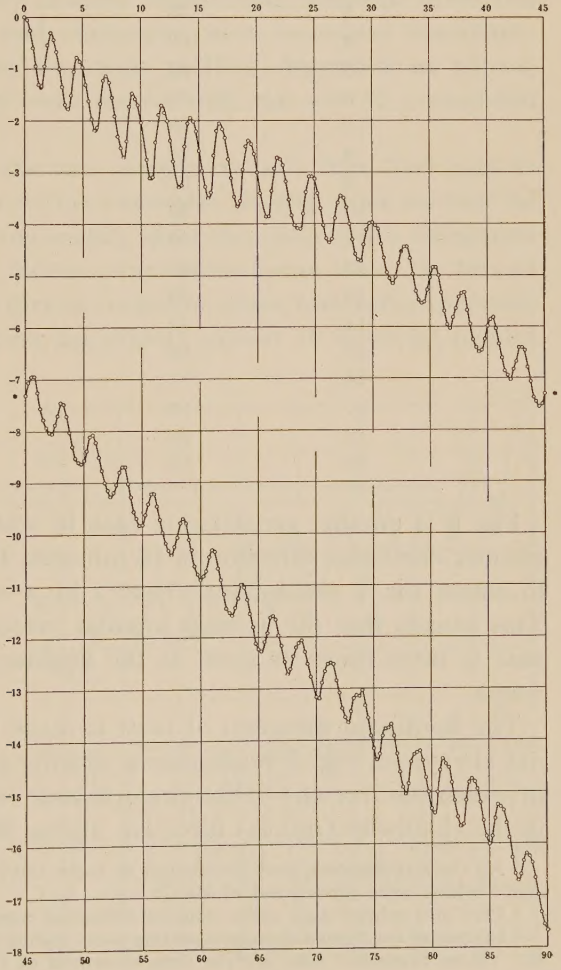

Fig. 7. Time course of rotation of the free end of the protoplasmic thread. The undulating curve declines wave by wave showing the dominance in clockwise rotation over counter-clockwise rotation. Length of the thread at the end of experiment: 45 mm. Temp.: 30°C.

revolutions around its own axis during a period of 20 minutes. This means that the thread twists itself irreversibly and practically unlimitedly in the counter-clockwise direction.

TABLE I

Rotation of the free end of the suspending protoplasmic thread.[1]

Exp. No.	Duration of experiment	No. of revolutions during experiment[2]	Length of thread[3]
	(min.)		(mm)
1	77	12.2	35
2	96	7.0	14
3	30	−0.4	31
4	100	−2.3	39
5	33	0.3	21
6	97	−19.2	45
7	88	−17.0	48
8	44	0.2	50
9	67	−1.8	80
10	33	−11.1	80
11	45	13.5	28
12	193	10.0	30
13	90	12.1	24
14	45	3.4	24
15	96	33.0	23
16	18	0.4	24
17	58	2.0	50
18	25	1.0	32
19	55	1.4	23

Fig. 6 is another remarkable case in which the tip rotated 9 times in the counter-clockwise direction in 16 minutes. Here we see that the tip continued to rotate for 4 revolutions (1440°) in a single stroke lasting 80 seconds. This means that the average angular velocity of the twisting motion in this case is three times as great as the angular velocity of a second hand of a watch.

The dominant direction of twist is more frequently counter-clockwise but not always so. Fig. 7 represents a striking example of the clockwise rotation in which the free end of the protoplasmic thread twisted for 17.7 revolutions in the clockwise (minus) direction during 90 minutes.

[1] All the experiments were conducted at room temperatures between 23–27°C except Nos. 6 and 7 which were carried out at 30°C.

[2] Plus and minus signs show counter-clockwise and clockwise rotation respectively.

[3] Length of the thread changes spontaneously during the experiment as will be stated in section (b). Values presented here are the ones measured at the end of the experiment.

The data so far obtained are collected in Table I where it is seen that 13 out of 19 experiments revealed the counter-clockwise tendency. The Table reveals that the strength of the twisting tendency is very different from specimen to specimen. It happens occasionally that the dominant direction of rotation reverses during the course of an experiment. Among the data listed in Table I, 6 specimens showed a conspicuous trend in the counter-clockwise direction and 3 specimens in the clockwise direction of rotation.

b) *Relation of elongation and contraction to torsion.* The motion of the hanging strand consists not only of torsion around its own axis but at the same time of elongation and contraction in the longitudinal direction. In order to determine if there is any definite relationship between these two kinds of motions, both rotation and elongation were measured simultaneously. Rotation was measured by the same method described as before, while elongation and contraction were traced with the aid of a modified horizontal microscope.

Simultaneous measurement of the two motions reveals that their periods are the same. Figs. 8–10 are some of the examples. In Fig. 8 we see that the troughs of the rotation waves approximately meet the crests of the elongation waves showing that the free end rotates counter-clockwise when the thread elongates and clockwise when the thread contracts. More precisely expressed, the maxima of the elongation waves lag slightly (about 10 seconds) behind

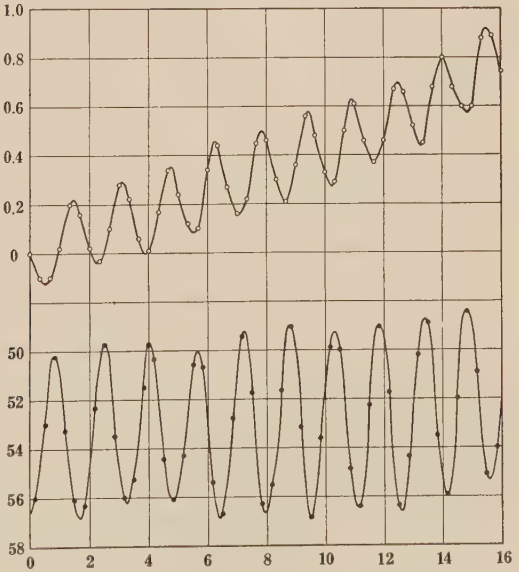

Fig. 8. Graphical representation of both rotation and elongation by means of simultaneous measurement. In this as well as in the following figures ordinates representing length of the thread are made upside down as the thread elongates downwards. Open circles: rotation; solid circles: length. Temp. 23.5°C.

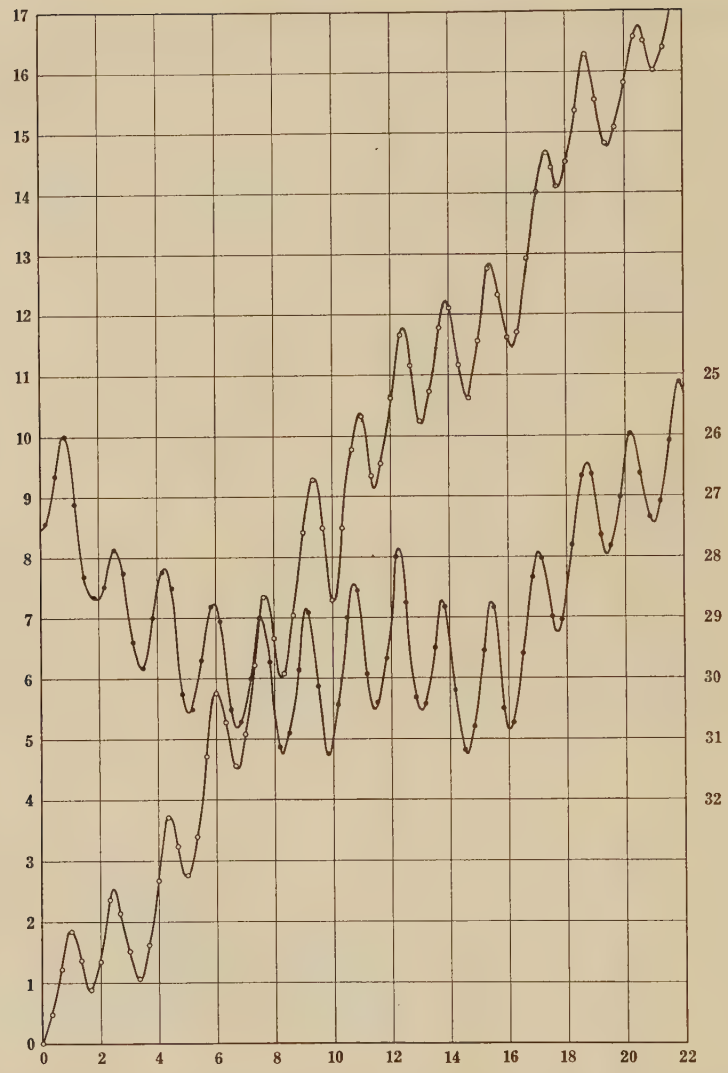

Fig. 9. Time course of rotation (open circles) of the free end of the thread and that of elongation and contraction of the thread (solid circles). Temp.: 27°C.

the minima of the rotation waves. The elongation and rotation waves here both have a period of 95 seconds.

In Fig. 9, the phase relation between rotation and elongation waves is the opposite of that in Fig. 8. Both maxima and minima of the two waves

in this case nearly meet each other, showing that the free end rotates counter-clockwise when the thread contracts and clockwise when the thread elongates.

Simultaneous measurements so far conducted do not demonstrate a constant and simple relationship between rotation and elongation. In Fig. 10 the torsion curve represents peculiar two-peaked waves while no corresponding pattern is seen in the elongation waves.

The magnitude of the tendency of a protoplasmic thread to twist in one direction or the other is not accompanied by any corresponding elongating or contracting tendency of the thread. Such tendencies are indicated by the steepness of the ascending or descending trends of the undulating curves, or, in other words, by the inclination of the imaginary axes of the undulating curves against the base-line. Though such axes of waves are not indicated in any of the figures in this paper, they can be obtained by the same method as that applied by Kamiya (2) when he traced the 'polarity line' of the motive force curve of protoplasmic flow.

To sum up, no clear correlation has been found between the rotation and elongation in respect to the phase, wave pattern, and ascending or descending tendency of the wave except that the periods of the two waves are exactly the same.

c) *The relation of protoplasmic streaming to torsion.* One could assume

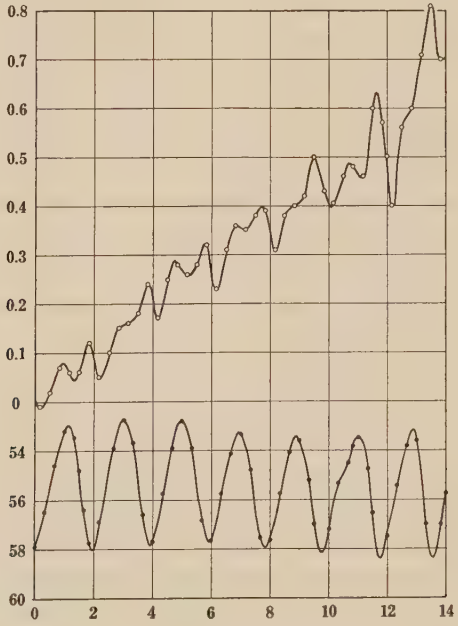

Fig. 10. Time course of rotation (open circles) of the free end of the thread and that of elongation and contraction of the thread (solid circles).

that the streaming of the protoplasm within the hanging thread plays an important role in bringing about the torsion of the thread. In order to detect a possible relationship between protoplasmic streaming and torsion, one must observe the upward and downward streaming in the thread and record successively the times of reversal of the streaming while measuring simultaneously the rotation of the free end of the thread.

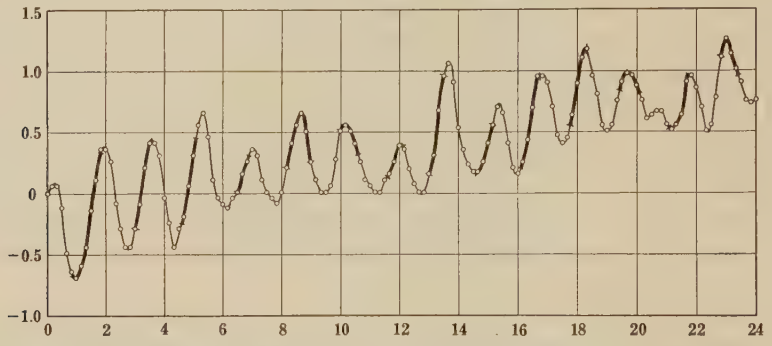

Fig. 11. Time course of rotation of the free end of the thread with indication of the streaming duration. Heavy line: duration of upward flow; thin line: duration of downward flow. Length of the thread at the end of experiment: 32 mm. Temp.: 24°C.

The streaming may be observed in the vertical thread near the upper terminal through a horizontal microscope. To improve visibility of the streaming, a small block of transparent plastic was applied to the thread locally.

Fig. 11 shows that the free end of the thread rotates in relation to time just as in the case of the other figures presented above. The undulating curve shows, as before, a tendency to ascend gradually, an indication of twisting more in a counter-clockwise direction. The portions indicated by the heavy line show the periods in which upward streaming took place along the vertical thread, and the portions indicated by the thin line show the periods in which downward streaming took place. So far as this figure is concerned, the lower tip of the thread shows the tendency to rotate more in a counter-clockwise direction when the protoplasm flows upward, and more in a clockwise direction when the protoplasm flows downward, yet this is by no means a strict rule even in this particular specimen.

In Fig. 12 are represented the torsion and change in length of the thread, and simultaneously the time durations of the upward and downward streaming. It is interesting to see that there are 4 reversals in the streaming direction during one period of twisting motion, i.e. two durations of upward flow and two alternate durations of downward flow. From this fact one might believe that the period of twisting motion must be twice as long as that of the proto-

plasmic streaming. But we know that such a situation may be brought about when the velocity curve of the streaming consists of two-peaked waves as a result of interference of component waves having different periods (3).

All in all, there seems to be no causal and directly coupled relation between the flow of the endoplasm and the torsion of the thread.

To prove this more strikingly, we conducted some experiments using the technique of Kamiya's double-chamber (2). In Fig. 13 is shown somewhat schematically the double-chamber consisting of compartments *A* and *B*. In *A* there is a protoplasmic blob (*a*), and in *B* a hanging strand (*b*) with a small mirror (*m*) attached to it. As is seen in the figure, the strand of

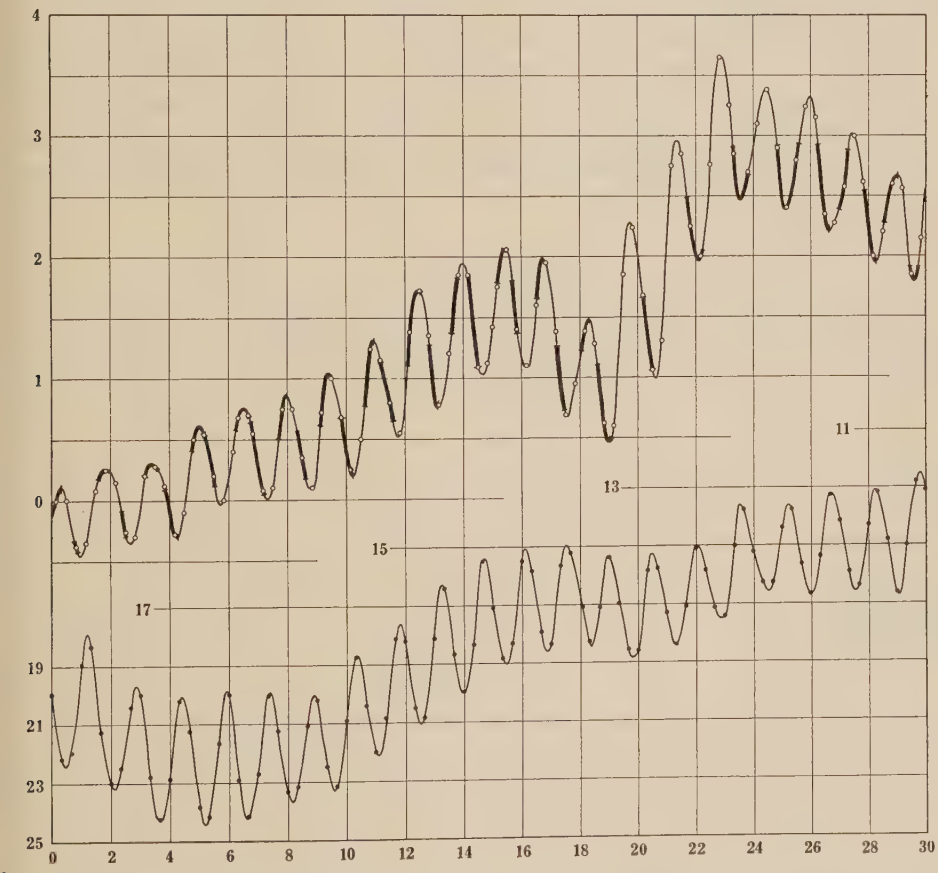

Fig. 12. Graphical representation of rotation (open circles) of the free end of the thread, of elongation of the thread (solid circles) and of the streaming duration in the thread. Heavy line on the rotation curve shows the duration in which the streaming took place upwardly in the thread while thin lines show the opposite case. Temp.: 26°C.

protoplasm (*b*) is connected with the protoplasmic mass (*a*) through the air-tight septum between the two compartments. If we apply compression or suction to the compartment *A* while compartment *B* is kept open to the atmosphere, the endoplasmic flow along the thread in the septum is strikingly affected. Thus, by changing the air pressure in *A* we can control the speed and direction of the endoplasmic flow inside the hanging strand very accurately. By this means it is possible to accelerate, stop or reverse the flow, and yet we find that such modifications of the flow exert no direct effect whatsoever on the twisting motion of the protoplasmic thread.

Special attention must also be paid to the fact that the pronounced twisting tendency, to the right or to the left, was often observed in very thin threads, in which the endoplasmic flow, if any, must be insignificant.

Thus, the observations and experiments so far conducted all show that the protoplasmic flow along the hanging thread is not responsible for the torsion of the thread. Coincidence of the period of streaming and the period of torsion, however, suggests that both types of motion are controlled by one and the same basic physiological rhythm.

DISCUSSION

When a protoplasmic thread is held by one end and hung vertically, it was demonstrated not only that the free end of the thread rotates to the right and left alternately around its own axis with a period of 2 minutes or so,

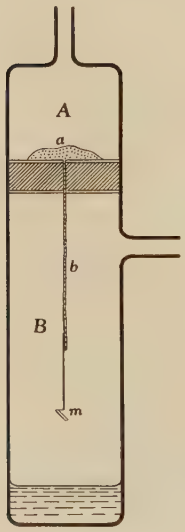

Fig. 13. Double-chamber for controlling the protoplasmic streaming into or out of the hanging thread by regulating the air pressure in compartment *A*. Compartment *B* opens to the atmosphere. *a*: protoplasmic mass. *b*: hanging thread of protoplasm. *m*: tiny, hanging mirror.

but also that the angle of rotation in one direction is always dominant over that in the other. The repetition of such a motion results in bringing about endless rotation of the free end in one direction or the other. The thread, therefore, behaves as if it were wound up in one direction. However, as the lower tip of the thread is free to rotate, it must naturally tend to assume the most stable orientation, or the orientation with minimum potential energy.

Once the thread is killed by whatever means, all the perceptible motions immediately stop. It is thus clear that some functioning of living protoplasm is responsible for this peculiar phenomenon. Such a behaviour of the living thread would not be intelligible were we not in a position to appreciate that the structural elements (submicroscopic fibrils) of protoplasm 'slip' against each other. Slippage of the structural components may take place by reconversion of junctions.

We now turn to the consideration of the fact that the thread supports a mirror, however small, besides its own weight. This means that the thread has considerable tensile strength, and consequently must, at least in part, be in the state of a stiff gel.

On the basis of the above facts we are led to the conclusion that in living protoplasm the process of reconversion of the linkage of structural elements, or sol-gel transformation at the submicroscopic level, is occurring locally even in the protoplasm which is apparently in a gelatinized state, as long as it is living.

The concept of simple reconversion of junctions, however, does not suffice to account for the dominance in rotation in one direction over that in the other, or, in short, dynamic anisotropy of the living matter. The point which must be stressed is therefore, the implication of the above fact that the repetition of a breaking-down and rebuilding process does not take place at random but proceeds according to a special scheme leading to a slippage of structural elements in a definite direction. If a similar process takes place in a blob of protoplasm, it may show a certain recognizable motion on the microscopical level which we call protoplasmic streaming or cyclosis.

In the present paper we have reported the twisting behaviour of the protoplasmic thread without subjecting the material to any special experimental control. The effect of various physical and chemical agents on the rotation of the free end of the living thread on the one hand and the approach to the submicroscopic structures of the thread by means of various optical means on the other may help better to understand the nature and mechanism of the phenomenon. How far the molecular configuration of the structural elements is causally related to the phenomenon is a problem to be dealt with.

SUMMARY

1. A short length of protoplasmic thread of the plasmodium of the myxomycete *Physarum polycephalum* hung in moist air between two supports often tends to take the form of a spiral or of loops. These forms strikingly resemble those assumed by a piece of string when it is twisted. The formation of a spiral or loops by a protoplasmic thread is believed to be due to torsion established in the living matter.

2. If the protoplasmic thread is hung vertically by one end while the other, lower, end is left free, the free end rotates to the right and left alternately with a nearly constant period of about 2 minutes at room temperatures. The angle of rotation of each stroke differs according to the specimen and to the time of measurement.

3. The angle of rotation of the free end in one direction is greater than that in the opposite direction in each successive motion. Hence, by repetition of the motion the free end of the thread continues to rotate in one direction unlimitedly.

4. The concept of slippage of the structural components in protoplasm was introduced in order to account for the unlimited rotation of the free end. Such a slippage is considered to take place by reconversion of junctions in the living protoplasm even though it is in that state commonly called a gel.

5. We have so far encountered a counter-clockwise rotation (as seen from above) more often than a clockwise rotation.

6. The hanging thread contracts and elongates longitudinally with the same period as does the twisting motion. However, no clear-cut relationship between rotation and longitudinal motion has been found.

7. The protoplasmic flow which takes place within the hanging thread does not play a role which is causally related to the twisting motion.

The present work was started by one of us (N. K.) as early as 1942 at the Biological Laboratory of Haverford College, Pa. Though the work carried out there was soon interrupted by his repatriation, it is his most pleasant duty to express his gratitude to Professor Howard K. Henry of Haverford College for his generosity in granting him the use of the facilities of the Biological Laboratory at the time of a most serious international situation.

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Experimental Cell Research, **6**, *17–29 (1954)*

A STUDY OF MICHEL'S FILM ON MEIOSIS IN *PSOPHUS STRIDULUS* L.[1]

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Received April 8, 1953

A NUMBER of studies of chromosome movement in mitosis and meiosis are now available (1, 2, 7, 9, 10, 15, 16). Most of the results reported so far give only data on chromosome separation in anaphase although Ris (16) has also measured the changes in spindle length and width in meiosis in the grasshopper *Chorthophaga viridifasciata*. It would be of great value for our understanding of the sequence of events in chromosome separation if simultaneous measurements of the spindle and cell diameters as well as chromosome separation were available. The measurement of a number of cell parameters can only be done readily on cinematographs of favorable material.

After seeing the excellent film on spermatogenesis in the grasshopper *Psophus stridulus* L. taken by Dr. Kurt Michel of the Zeiss Optische Werke, we decided to make such measurements on this film.

METHODS AND MATERIALS

The film used for this study was the U.S. Army film, PMF 5036, "Mechanisms of Cell Division" which was loaned to us through the kind offices of Dr. Edward M. Gunn, Chief, Medical Illustration Service of the Armed Forces Institute of Pathology. This is a copy on 16 mm film of the original film (35 mm) made by Dr. Kurt Michel. Dr. Michel has reported on the method of preparing the material and has made measurements of chromosome separation on the first primary spermatocyte division shown in the film (13). Since this reference is not readily available and since Dr. Michel has very courteously given us some further information in a personal communication, the methods and materials used by him are summarized below. The testes of *Psophus stridulus* L. were obtained by a technique similar to that used by Belar (2). Testis follicles were teased apart in "Normosallösung" on a cover slip and the cover slip inverted on a well slide, the well containing pure paraffin oil. All procedures were carried out at a room temperature of about 20°C. A Zeiss achromatic phase contrast

[1] This project has been aided by an Institutional Research Grant from the American Cancer Society.

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objective (N.A. 0.65, 40×) was used. Pictures were taken at a frequency of eight frames per minute on AGFA-aerochrom 35 mm film with an exposure time of about one second. A green filter was used. The final magnification on the film was 200×.

A comparison of the pictures in reference 13 with the 16 mm. film available to us indicated that the magnification on the 16 mm. frames was about 100×. Guides for the 16 mm. film were attached to the stage of a Bausch and Lomb VH microprojector and the film projected on a table top at a magnification of 11.7×. On every 16th frame, the outlines of the cell, chromosomes and spindle area were traced on paper. A copy of one of the tracings made during metaphase is shown in the lower left hand corner of Figs. 1, 2 and 3.

The spindle area appears as a somewhat lighter area surrounded by a cuff of mitochondria. The transverse diameter of the spindle was measured as the maximum inner diameter of the mitochondrial cuff at the equator. The boundary between the mitochondria and the spindle area is usually quite sharp at the equator so that this measurement is clearly defined. The mitochondria usually do not extend around the poles. Thus the transition from the light spindle area to the surrounding cytoplasm is more gradual and the choice of boundaries for spindle length correspondingly more difficult. At times it became too difficult to decide on the boundaries for the spindle length (Fig. 1), and no attempt was made to trace the boundaries. It is obvious from the above discussion that if one were to make measurements of spindle length directly on the projected pictures, one might become biased in the choice of boundaries after a few measurements had been made. It was primarily for this reason that all outlines were first traced on paper and the measurements then made on the tracings.

Measurements of chromosome separation were made only on those chromosomes that were sharply outlined throughout most of a division. Chromosome separation was measured as the distance between the leading ends of the separating chromosomes. These measurements are given by closed circles and crosses in the figures. It will be seen that open circles replace closed circles and crosses towards the end of each division. This signifies that the leading ends of the chromosomes that were being measured were no longer clearly defined and that all the chromosomes were becoming clumped near the poles. The open circles therefore represent separation of the chromosome groups in late anaphase and telophase, the measurement being the maximum distance between the two groups measured along a line through their centers.

The error in the measurement of the cell diameters is probably less than one micron. The error in the measurement of chromosome separation and the transverse diameter of the spindle is probably also on the order of one micron, although it might be somewhat more for the latter. However, the error in the measurement of spindle length could be up to 4 μ at times.

Changes in cell volume are given for the second primary spermatocyte division in the film. This cell remained symmetrical in appearance up to the formation of the cleavage furrow. The volume is calculated on the assumption that the cell is an ellipsoid of revolution about the spindle axis. Assuming an error of one micron in the measurements of the cell diameters, we get an error of about $4.5–5 \times 10^3$ cubic micra in the calculated cell volume.

Four spermatocyte divisions are shown in the film, two of primary and two of secondary spermatocytes. These will be discussed in the order in which they appear in the film.

RESULTS AND DISCUSSION

Fig. 1 shows the changes in cell and spindle diameters and the course of chromosome separation for two chromosomes in the first primary spermatocyte division shown in the film. The measurements of spindle length were discontinued near the end of metaphase because the boundaries were no longer clearly defined. The cell was not symmetrical in shape because of

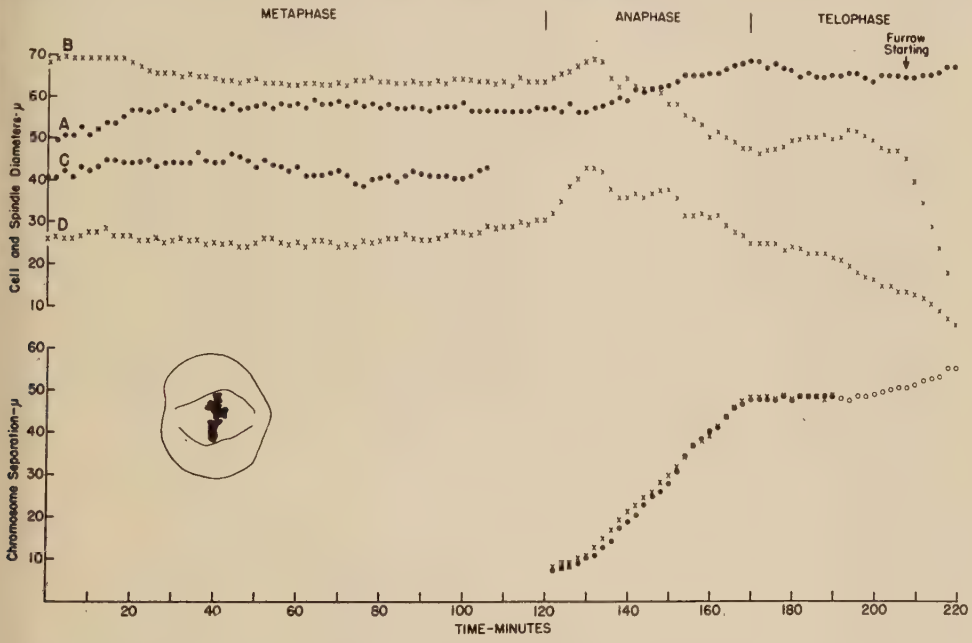

Fig. 1. Primary spermatocyte division. A. Cell diameter along spindle axis. B. Transverse diameter of the cell. C. Spindle length. D. Transverse diameter of the spindle.

pressure from surrounding cells. The change in cell diameters at the beginning of Fig. 1 was probably due to a shifting of the surrounding cells. Chromosome separation started almost simultaneously with an increase in transverse diameters of the cell and spindle. The latter is very striking when seen in the film. Just as chromosome separation starts, there is a bulging out of the mitochondria around the equatorial plate. In this division, there is no suggestion of a decrease in cell length accompanying the equatorial bulging although this occurs in the other primary spermatocyte division. The length of the cell begins to increase after chromosome separation has started.

The course of the second primary spermatocyte division in the film is

shown in Fig. 2. This cell was the only one which was symmetrical throughout division and did not have any other cells pressing on it. The changes in cell and spindle diameters that accompany chromosome separation are quite marked. The increase in transverse diameters of cell and spindle and the decrease in longitudinal diameters just precede chromosome movement. This bulging out of the spindle at the equator suggests a contractile mechanism. If so, the increase in transverse diameter of the cell and decrease in cell length would suggest some connection of the spindle poles to the overlying cell cortex.

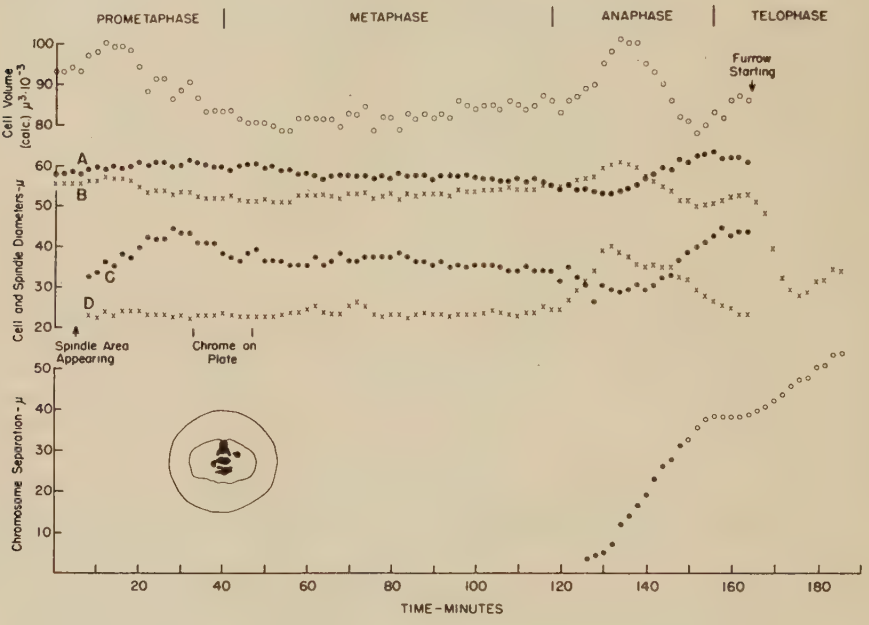

Fig. 2. Primary spermatocyte division. A, B, C, D as in Fig. 1.

The decrease in length of the spindle is a little larger than our estimate of the maximum error in this measurement. The changes in the other measurements are much larger than the errors involved. This also applies to the changes in cell volume provided that the assumption that the cell was an ellipsoid of revolution around the spindle axis holds. Assuming that this is justified, it may be significant that a change in cell volume occurs in prometaphase and anaphase, both stages in which there is chromosome movement. In the former the chromosomes are being lined up at the plate and in the latter they are separating. The volume change in anaphase is

remarkably large, being about 20×10^3 cubic micra. The cell surface increases by about 2×10^3 square micra. Since the surface tension of most cells lies in the range 0.1–1 dyne/cm (5), the cell must perform at least $0.2–2 \times 10^{-5}$ ergs of work during this increase in volume. The simplest assumption might be that there is an increase in the osmotic pressure of the cell due to breakdown of some cellular substances into smaller components and a resulting uptake of water. However, since the volume increase in anaphase is associated with movement of the chromosomes to the spindle poles (spindle elongation does not begin until the dilatation of the cell has passed its peak), it may well be more to the point to attempt to correlate the volume increase with the processes accompanying shortening of the chromosomal fibers. We would then require a set of reactions which have a net positive partial molal volume change throughout this process. Since a contractile mechanism may well be involved, the first reactions that come to mind are those involved in muscle contraction. Hydrolysis of adenosinetriphosphate and creatine phosphate is accompanied by volume constrictions of 10 and 11.5 cc/mole respectively but there is a volume dilatation of 24 cc per mole lactic acid formed from glycogen (12). Under anaerobic conditions, muscle contraction is accompanied by an initial volume constriction due to hydrolysis of adenosinetriphosphate and creatine phosphate, followed

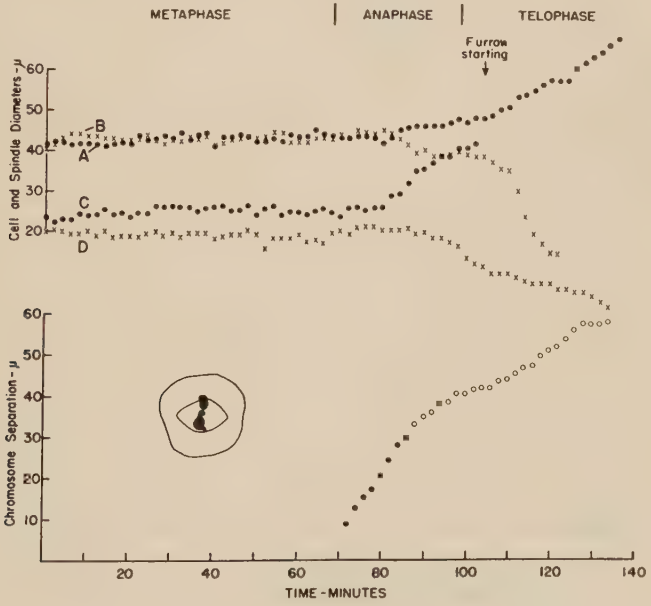

Fig. 3. Secondary spermatocyte division. A, B, C, D as in Fig. 1.

by a slower volume dilatation due to lactic acid formation. The volume does not return to the baseline, but remains at the increased level after the contraction. There is also the possibility of sol-gel changes since many of the cytoplasmic sols show volume increases on gelation (11). All of these considerations should be kept in mind, but we do not wish to put much emphasis on this since it comes from only one observation. A more detailed discussion is best deferred until more observations are available.

Chromosome separation consists of three phases. The first two overlap to give the anaphase separation. The first part of anaphase movement occurs with little change in spindle length. The spindle length decreases just before chromosome separation starts and then remains constant in the first part of anaphase movement. The chromosome movement in this part is mostly movement to the spindle poles without change in spindle length. On the other hand, most of the movement in late anaphase can be accounted for by the increase in spindle length. This can be readily seen by subtracting the increase in spindle length in late anaphase from the chromosome separation curve. There is then a period of rest in early telophase followed by further separation of the chromosome groups accompanying the advance of the cleavage furrow. By this time the chromosomes are clumped in the telophase

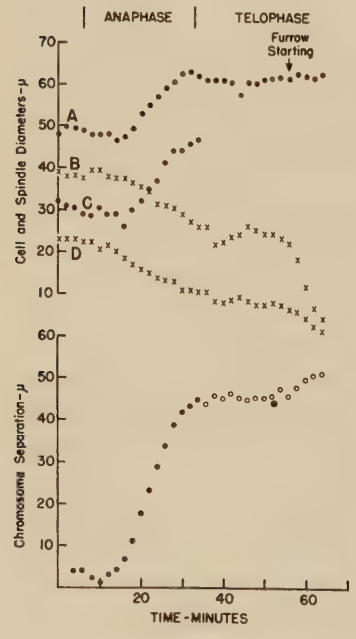

Fig. 4. Secondary spermatocyte division. A, B, C, D, as in Fig. 1.

groups and may no longer be attached to the spindle. The spindle is constricted by the cleavage furrow and continues to lengthen. These late changes in the spindle may be just a reflection of cytoplasmic action in cleaving the cell.

The type of anaphase movement in which spindle elongation does not start until the chromosomes have moved a considerable distance to the poles has also been found by Ris (16) in the spermatocytes of the grasshopper *Chorthophaga viridifasciata*. Ris (16) has also shown an increase in spindle width in *C. viridifasciata*, but in his curves the increase occurs after chromosome separation has started.

The course of the two secondary spermatocyte divisions is shown in Figs. 3 and 4. Unlike the primary spermatocyte divisions, there are no marked changes in cell and spindle diameters when chromosome separation starts. Otherwise, these curves do not differ much from those of the primary spermatocytes. There is one interesting feature in Fig. 4. The chromosomes measured were two rod-shaped chromosomes lying side by side but distinctly separate at the equatorial plate. Just before chromosome separation, these two homologs came together and then began to unfurl at one end.

ANALYSIS OF CHROMOSOME MOVEMENT

From the above discussion, it can be seen that chromosome movement in anaphase in *Psophus stridulus* L. is separable into a) movement of the chromosomes to the poles (contraction of spindle fibers?), and, b) elongation of the spindle. Both of these give fairly symmetrical sigmoid curves of chromosome separation. The full curve of chromosome separation obtained by adding these two components is almost sigmoid but tends to show this biphasic character. Barber (1) has given sigmoid curves of chromosome separation for *Tradescantia* in which there is no spindle elongation. Ris (15, 16) has given sigmoid curves of spindle elongation and chromosomal separation for primary spermatocytes of *Protenor* and *Thelia*, spermatogonia of *Protenor*, secondary spermatocytes and embryonic cells of *Tamalia* and primary and secondary spermatocytes of *C. viridifasciata*. Chromosome separation in the primary spermatocytes of *Tamalia* is apparently entirely due to spindle elongation (15). Hughes and Swann (10) give a sigmoid curve for spindle elongation in chick fibroblasts and a curve of chromosome separation which has only a very small sigmoid inflection at the beginning of the curve (7). Fell and Hughes (7) give generally sigmoid-shaped curves for overall chromosome separation in mouse fibroblasts, and Hughes and

Preston (9) have given sigmoid curves for chromosome separation in fibroblasts of *Rana temporaria, Xenopus laevis* and *Triton cristata.* Insofar as chromosome separation due to movement to the poles is concerned, it is in the nature of the case that the initial portion of the curve must be concave upwards because the chromosomes start from rest, and the final portion of the curve must be concave downwards because the chromosomes come to rest with respect to the spindle poles. Since physical processes are continuous, the condition of continuity and the boundary conditions (*i.e.*, the chromosomes are at rest for some short interval of time before separation starts and for some interval after they reach the poles) determine the general shape of the chromosome separation and velocity curves for some short intervals at the beginning and end of chromosome movement to the poles. Thus not only must the initial part of the chromosome separation curve be concave upwards and the final portion be concave downwards, but the initial and final portions of the velocity-time curve must be concave upwards. There is of course no *a priori* reason for the middle portion of this curve to be as it is; it might just as well have a number of oscillations as a smooth curve connecting the initial and final portions.

In any analysis of chromosome movement it is desirable to separate movement due to spindle elongation and movement to the poles, since these probably involve different mechanisms. Chromosome movement in certain cells may be entirely due to one of these factors. They are separable experimentally as well as analytically because spindle elongation can be inhibited by chloral hydrate (16) without affecting shortening of chromosomal fibers. This strengthens the argument that these are really two different processes. In the analysis presented below, we will restrict ourselves to that part of anaphase separation due to movement of chromosomes to the poles since definite hypotheses are available for testing for this phase of chromosome movement. The favored hypothesis is that the chromosomes and spindle poles are connected by spindle fibers which contract to pull the chromosomes to the poles. The evidence for this has been summarized by Cornman (3) and has been further discussed by Hughes and Swann (10). Of course any such analysis cannot provide proof for the contractile hypothesis. It can tell us whether the kinetics of chromosome movement is compatible with a contractile mechanism or an alternative hypothesis such as a mutual repulsion of the chromosomes under a force that varies as an inverse power of the distance.

We have been able to fit the curve of chromosome movement to the poles quite well with the integrated curve of the Gaussian probability function.

This is not unique; any symmetrical sigmoid function such as K arc tan x/c could be fitted equally well. In this analysis we will not use any of these functions, since the analysis depends only on the general properties of functions that are sigmoid-shaped and not on the specific functions which may be used to represent the curves analytically.

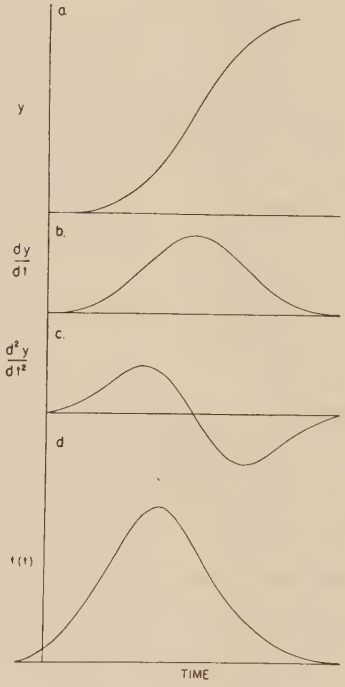

Fig. 5. Curves of chromosome separation (movement to the poles), velocity, acceleration and the force function $f(t)$. Curves a, b and c are to scale.

Assume that the curve of chromosome movement due to movement to the poles is approximately a symmetrical sigmoid curve as in Fig. 5a. Then the curves of chromosome velocity and acceleration are as shown in Figs. 5b and 5c. Let a time function $f(t)$ represent the forces tending to move the chromosomes to the poles and $r(v, t)$ represent the viscous drag opposing movement. There is little loss in generality if we assume that $r(v, t)$ is separable,

1) $$r(v, t) = h(t)\, g(v).$$

Then:

2) $$\frac{m\, d^2 y}{d t^2} = f(t) - h(t)\, g(v)$$

where m is the chromosome mass, v the velocity, y the distance and t time. If we knew the nature of $h(t)\, g(v)$, we could find $f(t)$. For the low velocities involved, we may assume that,

3) $$h(t)\, g(v) = h(t)\, v$$

and that the variation in $h(t)$ will not be very large in the course of chromosome movement. For Newtonian systems at these low velocities, $h(t) = k$, where k is the frictional coefficient. Let us first treat this as though equation 4 held rigorously and then see what modifications are introduced by the non-Newtonian nature of protoplasm.

4) $$f(t) = m \frac{d^2 y}{d t^2} + k \frac{d y}{d t}$$

For a spherical particle of diameter b moving in a fluid of viscosity η, Stokes' formula gives,

$$k_0 = 3 \pi \eta b.$$

For a rod-shaped particle like the chromosomes of *P. stridulus* which we have measured, $k > k_0$.

Since the spindle is a colloidal system, it is undoubtedly non-Newtonian in behavior. However, we can obtain a lower bound for $h(t)$ and an upper bound for m and assess the relative importance of the terms in $\frac{d^2 y}{d t^2}$ and $\frac{d y}{d t}$ in equation 4. Cytoplasmic viscosity values have been found to fall in the range .01–.1 poise (8). A lower bound for the viscosity of the spindle will certainly be .01 poise, the viscosity of water. The actual value may well be .1 poise or larger. The rod-shaped chromosomes in *P. stridulus* have dimensions of about $1.7 \times 1.7 \times 10$ micra. Taking one micron as the lower bound for b,

$$h(t) > 3 \pi \cdot 1 \cdot 10^{-4} \cdot 10^{-2}$$

$$\therefore h(t) > 10^{-5} \text{ (gm sec.}^{-1}\text{)}.$$

The volume of one of these chromosomes is about $3 \cdot 10^{-11}$ cm^3. The density is certainly less than 1.5 gm/cm^3. Therefore an upper bound for m is,

$$m < 10^{-10} \text{ gm}.$$

Therefore a lower bound for $h(t)/m$ *is* 10^5.

$$\frac{h(t)}{m} > 10^5.$$

It follows that if $h(t)$ were a constant, $h(t) = k$, the shape of $f(t)$ would be practically the same as that of $\frac{dy}{dt}$ since $\frac{dy}{dt}$ and $\frac{d^2y}{dt^2}$ are of the same order of magnitude.

5) $$\therefore f(t) \simeq k \frac{dy}{dt}$$

This conclusion would still hold if $h(t)/m = 10$, so we have a wide margin of safety in the above estimate. We may summarize this argument in a more intuitive form as follows; for a particle moving through a viscous fluid (not necessarily Newtonian) at low velocities, if the viscous drag is much larger than the mass, $h(t) \gg m$, the force required to move the particle will be determined almost entirely by the viscous drag and only slightly by the inertia of the particle.

The non-Newtonian nature of protoplasm will modify this somewhat. Viscosity measurements show that solutions of asymmetric protein molecules are usually thixotropic. Myosin for example shows thixotropic behavior (6). Similarly all measurements of protoplasmic viscosity show that protoplasm is thixotropic (17). Thixotropic substances, unlike Newtonian fluids, exhibit a yield value (*i.e.* when a force is applied to a particle in such a medium, the particle does not begin to move until a certain finite force is acting on the particle). Therefore we may reasonably expect that the curve of $f(t)$ starts before that of dy/dt. From the thixotropic nature of protoplasm, we may also expect that $h(t)$ will be relatively constant or decrease in the course of chromosome movement. Taking these factors into account, $f(t)$ will start before dy/dt and may be skewed to the left compared to dy/dt giving a curve somewhat like Fig. 5d. A short range attractive force holding the chromosomes together at the equatorial plate would be equivalent to a yield value and could be included in the above considerations. One more factor should be taken into account. Protoplasm shows not only an anomolous viscosity but also a visco-elastic behavior. Thus if a particle in protoplasm is displaced, it returns part way on release of the force. Crick and Hughes (4) have shown that when an iron particle in a chick fibroblast is rotated by a magnetic field, there is some elastic return. This elastic behavior is however not great (17) and we may therefore expect that the contribution of this to $f(t)$ will also be small compared to $h(t)\, dy/dt$.

The curve of the force acting on a chromosome to give motion to the

spindle poles will thus be generally like Fig. 5d. If the curve of chromosome separation is not as symmetrical as in *P. stridulus*, the general shape of $f(t)$ will remain the same but the peak may be shifted to the right or left in comparison to the case discussed. The curve of $f(t)$ is compatible with a contractile mechanism but certainly not compatible with a force function which varies as an inverse power of the separation. Hughes and Swann (10) have come to the same conclusion in their study of chromosome movement in chick fibroblasts. Excluding the initial and terminal phases of chromosome movement, they obtained an approximately linear relationship between chromosome velocity and separation. As they point out in their paper, this may be a coincidence because their curve includes the effects of movement to the poles and spindle lengthening. If only movement to the poles is considered, the relationship is not linear. This is obvious if the initial and terminal phases with their necessary boundary conditions are included in the analysis. The initial sigmoid portion of the curves of chromosome separation in chick fibroblasts is very short and the final portion is prolonged giving a very unsymmetrical sigmoid curve. The velocity-time curve would therefore be skewed to the left compared to Fig. 5b. This would result in a similar change in $f(t)$. Otherwise the analysis given above remains the same.

For the sake of simplicity, we have treated movement to the spindle poles as if spindle lengthening did not add any significant terms to the equation of motion. In those cases in which chromosome movement is entirely movement to the poles or in which movement to the poles and spindle lengthening are entirely separate temporally, the argument stands unchanged. This is also true if the chromosomes move entirely in the spindle. However, in the case of chromosomes that project into the cytoplasm, if spindle lengthening involves movement of the chromosomes through the cytoplasm, an additional viscous drag term proportional to the velocity of spindle elongation must be added to equation 4. This would add another force in the final phase of poleward movement such that $f(t) \neq 0$ when the chromosomes reach the poles. However in *P. stridulus* the curves of spindle and cell elongation are very similar suggesting that the cytoplasm around the spindle is being deformed in the same direction and at about the same rate as the spindle. This would make the extra term small.

Rashevsky (14) has given an analysis of anaphase movement in which he assumes that an elastic force obeying Hooke's law pulls the chromosomes to the poles and that this is opposed by a diffusion force. There seems to be little justification for the assumption that the contractile force follows Hooke's law. Certainly if one were to assume a contractile mechanism, it would be

preferable to suppose that the force function follows a curve similar to that of a known contractile system such as muscle. Rashevsky's equation fits the curves of chromosome movement in the secondary spermatocytes of *Stenobothrus* fairly well. However the curves used were those Barber (1) obtained from measurements of photographs in Belar's paper and the points are too far apart to show an initial sigmoid portion. The equation does not fit the necessary boundary conditions at the start of chromosome movement.

SUMMARY

1. Chromosome separation and the changes in cell and spindle diameters have been measured in cinematographs of two primary and two secondary spermatocyte divisions in *Psophus stridulus* L.

2. Both primary spermatocyte divisions showed an increase in transverse diameters of the cell and spindle at the beginning of chromosome separation. In one of these there was also a concomitant decrease in longitudinal diameters and an increase in cell volume.

3. An analysis of chromosome movement to the poles gives a force function which is compatible with the hypothesis of a contractile mechanism.

The authors wish to thank Dr. A. R. T. Denues of the Sloan-Kettering Inst. for his interest and helpful criticisms in many discussions during the course of this work.

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CELL FRACTIONATION OF NORMAL AND MALIGNANT TISSUES[1]

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Received April 15, 1953

The method of cell fractionation by differential centrifugation has been applied to the study of many normal and malignant tissues, mainly to determine the intracellular distribution of enzyme systems (25, 30), but a comparative study of the proportions of the cell fractions in various tissues has so far not been reported.

The present series of cell fractionations was undertaken to explore the variability of the intracellular distribution of protein and nucleic acids among normal and malignant tissues, and to inquire into the relationship of various tumor types to the normal tissues studied.

MATERIAL AND METHODS

Tissues for fractionation were obtained from rats, mice, and humans. The normal rat tissues included liver, kidney, thymus, pancreas, and submaxillary gland; the tumors from rats included the Flexner-Jobling carcinoma, Walker 256 carcinosarcoma, the Jensen sarcoma, and liver tumors induced by three different carcinogens. Normal mouse liver, and the following mouse tumors were fractionated: a transplantable mammary adenocarcinoma, a transplantable adrenal carcinoma, and a transplantable lymphosarcoma. A human adrenal gland and two human tumors were also fractionated: a fibrosarcoma and a hypernephroma.

For the study of normal tissues and of primary rat tumors, male albino rats of the Sprague-Dawley strain were used; female rats of this strain were used as hosts for the transplantable rat tumors. They were fed a commercial dog chow supplemented with a whole grain diet, and water, *ad libitum*. Food in all cases was available to the animals until the time they were killed.

The mouse livers were obtained from female Rockland strain white mice; C_3H mice of both sexes were used as hosts for the mammary adenocarcinoma and lymphosarcoma; castrated Ce or F_1 hybrid Ce mice were the hosts for the adrenal car-

[1] This work was carried out during tenure of fellowships of the National Cancer Institute, U.S. Public Health Service, and of the American Cancer Society. It was supported in part by the Alexander and Margaret Stewart Trust. This paper is part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology at the University of Wisconsin.

cinoma. The mice were fed Purina Laboratory Chow, and food was available to the animals until they were killed.

Experimental animals were routinely killed with ether. The tissues to be fractionated were immediately removed and chilled, and all further operations were carried out in a cold room at 0 to 5°C. The liver and kidney were perfused, but the other tissues were used without previous perfusion. Ice-cold 0.85 M NaCl was the perfusion medium in the fractionations of rat kidney and mouse liver. Isotonic sucrose was the perfusion medium in the two fractionations of rat liver reported here. The tissues were minced by pressing through a lucite sieve, to remove as much as possible of the connective tissue. The tissue mince was homogenized in a plastic Potter-Elvehjem apparatus in a concentration of 12 per cent by weight in 0.88 M sucrose. The homogenate was separated by differential centrifugation into nuclear and mitochondrial, the microsomes were sedimented at 120 000 × g fractions as previously described (21, 22, 23). A minor modification was made in the case of pancreas and submaxillary gland, in which the high concentration of microsomes made it necessary to wash the mitochondrial pellet two times instead of the usual once. It has been recognized that the mitochondrial fraction obtained by the technic of Hogeboom *et al.* (9) consists of a lower, more densely packed, tan layer and an upper, pinkish fluffy layer (16, 19, 30). In the present study the fluffy layer has been included with the mitochondrial fraction. This appears to be justified by the results of a recent investigation which demonstrated that this fluffy layer material is predominantly mitochondrial in nature (12).

The whole homogenate and the cell fractions were analyzed for protein-nitrogen by a Nesslerization procedure (32) and for pentose and desoxypentosenucleic acids by Schneider's method (26). Recoveries were generally within 5 per cent of the total values found on analysis of the unfractionated homogenate.

Nuclear counts were made on the whole homogenate in a 1:40 dilution in 3 per cent acetic acid containing gentian violet in a final concentration of 1:10 000. The analytical values were expressed in terms of the average cell. Counts of separate dilutions of nuclei from a single tissue were consistently reproducible within ±5 per cent, and rarely varied more than ±2 per cent. Clumping of nuclei seldom occurred, and then only in mild degree, except for the thymus, where clumping was so severe as to preclude accurate counting (see below). The weight of the average cell as given in the table was calculated as the reciprocal of the number of nuclei per gram.

The primary carcinomas include rat liver tumors induced by three different carcinogens, and a human hypernephroma. The liver tumors were induced by two azo dye derivatives, 3′-methyl-4-dimethylaminoazobenzene and 4′-fluoro-4-dimethylaminoazobenzene, and by 2-acetylaminofluorene. The carcinogens were mixed with the diet, in concentrations of .058 per cent, .058 per cent, and .036 per cent respectively. Tumors were harvested after 3 to 5 months of treatment with the carcinogen. Only small, firm, grossly non-necrotic tumors were fractionated.

The two human tumors were obtained at surgery. They were chilled immediately upon removal, and were kept chilled throughout the fractionation procedure. In the case of the hypernephroma, the surgical specimen included the entire kidney with its tumor, and the adjacent adrenal. The tumor was a large mass, with many foci of necrosis; only grossly non-necrotic tissue was used for fractionation. The adrenal gland and kidney tumor were fractionated simultaneously.

The other human tumor was a fibrosarcoma of the thigh; it was the only primary sarcoma to be fractionated. It was a single large mass, measuring about 6 cm in diameter. Very little necrosis was apparent on gross or microscopic examination.

The Flexner-Jobling carcinoma, the Walker 256 carcinosarcoma, and the Jensen sarcoma were implanted subcutaneously in multiple sites in female rats of the Sprague-Dawley strain. They were harvested after 7 to 10 days of growth; each tumor weighed 1 gram or less. The few small foci of necrosis which were apparent on gross examination were carefully trimmed away. In the first fractionation of the Walker 256 tumor only 1.25 grams of tumor was fractionated; this is apparently too little to provide enough mitochondria for accurate analysis. Three grams were fractionated in the second experiment.

The adrenal carcinomas were originally induced by castration of Ce mice in the laboratory of Dr. G. W. Woolley, of the Sloan-Kettering Institute for Cancer Research, New York; they were maintained in this laboratory in castrated Ce and F_1 hybrid Ce mice. In the first experiment two tumors of independent origin, each in the first transplant generation, were fractionated together. In the second experiment, the second transplant generation of one of the previously fractionated tumor strains was analyzed.

The mammary adenocarcinoma originated in a C_3H mouse of the Andervont sub-line (2), and has been maintained in this laboratory in male and female $C_3H(H)$ mice for about two years. This tumor contains the mammary tumor milk agent. For fractionation, tumor was harvested from five mice. It was soft and hemorrhagic, and non-necrotic tumor material was selected only with difficulty.

The Gardner lymphosarcoma of the mouse (6C3HED) has been maintained in C_3H mice in this laboratory for many transplant generations. For fractionation, tumor tissue was implanted subcutaneously at multiple sites, in C_3H mice, and was harvested in 15 days. Extension into underlying muscle is common in this tumor. Hardly any necrosis was evident on gross or microscopic examination, but the neighboring muscle tissue had to be dissected away very carefully to avoid contamination with normal tissue.

RESULTS

The results have been presented in two ways. The numerical results are given in Tables I–IV, as quantities of protein-nitrogen and nucleic acid per average cell, and as quantities related to cell size. The results have also been plotted as "biochemical profiles", Figs. 1 and 2.

The average cell of each tissue had a characteristic and reproducible weight. The rat tumors varied no more in this respect than a single normal tissue. The mouse cells, normal and malignant, were smaller, and the human cells were larger than the corresponding rat cells.

The DNA per average nucleus in the normal tissues fell in a fairly narrow range, averaging about $10.5\ g \times 10^{-12}$. Among the tumors the variation in the DNA content of the average nucleus was not greater than among the

normal tissues studied, and the average value was in the same range as for the normal tissues.

In the fractionation of two of the tumors, the Flexner-Jobling carcinoma of the rat and the lymphosarcoma of the mouse, the DNA content of the average nucleus was very high as first determined. That the high values obtained were in error due to fragmentation of nuclei was suggested by the following procedure: An ascites suspension of the tumor cells was analyzed for DNA. Homogenization was not necessary, and the suspension was precipitated directly by 10 per cent trichloroacetic acid. Cell counts were done on suitable dilutions of the ascites suspension in 3 per cent acetic acid. The DNA per average nucleus in this experiment was found for the Flexner-Jobling tumor to be 11.6, and for the lymphosarcoma to be 8.3 g $\times$ 10^{-12}, compared to values of 10.7 and 10.4 g $\times$ 10^{-12} obtained on homogenates of the liver cells of the host animals. Therefore, the number of cells per gram of tumor for the two fractionations has been recalculated, assuming values for the DNA per nucleus of 11.6 for the Flexner-Jobling, and 8.3 g $\times$ 10^{-12} for the lymphosarcoma (as found for the ascites suspensions), and the other analytical results reported in the table have been calculated on the basis of the new estimates of the number of nuclei per gram.

In the thymus and submaxillary gland, also, the nuclear counts were not valid. In the case of thymus, the nuclei clumped too badly to permit accurate counting, and in the case of submaxillary gland, an excessively high value for the DNA content of the average nucleus, associated with the presence of some DNA in the cytoplasmic fractions, suggested that some of the nuclei had been fragmented during homogenization. Therefore the number of nuclei per gram was estimated on the assumption that the DNA content of the average nucleus is similar to that of other normal tissues, about 10.5 g $\times$ 10^{-12}. (The DNA content of single diploid nuclei of thymus has been found (31) to be similar to that of other tissues.) This estimate of the number of nuclei per gram was used to calculate the other figures for these tissues given in Table I. Errors introduced by the above assumption would result in errors in the figures for cell size and for the absolute quantities of protein and nucleic acid in each fraction, but would not affect the pattern of distribution of these components within the cell, nor their relation to cell size.

A high proportion of the DNA was recovered in the cytoplasmic fractions of pancreas also (about 30 per cent), but since the nuclear counts gave values for the DNA per average nucleus which were in the normal range, fragmentation of nuclei was not probable. It is possible that some of the DNA-nucleoprotein was extracted from the nuclei into the sucrose medium.

Considerable variation was observed in the RNA content of the average nucleus. In general, the tumor and liver cells contained much more than did the other tissues. Although the concentration of nuclear RNA with respect to cell size differed among the tumors on the one hand and the normal tissues on the other, the tumor range was distinctly higher than that of the normal tissues.

TABLE I

Protein-nitrogen and nucleic acid contents of the average cells of various normal tissues.

	No. of animals[1]	Nuclei per gram $\times 10^6$	Wt. of av. cell ($g \times 10^{-9}$)	DNA, av. nucleus ($g \times 10^{-12}$)	RNA ($g \times 10^{-12}$)				Protein-N ($g \times 10^{-12}$)			
					Nucleus	Mitochondria	Microsomes	Supernate	Nucleus	Mitochondria	Microsomes	Supernate
Liver (rat)	4	143	7.0	11.5	4.8	13.4	22.7	7.3	20.2	50.1	28.3	47.2
	4	140	7.2	12.8	6.2	14.0	21.4	10.4	26.6	48.0	26.6	58.0
Liver (mouse)	4	177	5.6	12.3	3.7	12.8	9.3	6.7	19.5	30.0	10.0	35.0
Kidney (rat)	4	326	3.1	9.5	1.4	1.3	3.1	4.0	10.0	16.1	10.8	25.0
	3	347	2.9	9.0	1.2	1.4	2.5	3.0	8.7	13.7	8.5	20.8
Thymus (rat)[2]	10	2 860	0.35	10.5	(0)[3]	.04	.57	.95	3.5	.16	.46	1.9
	9	2 800	0.36	10.5	(.30)[3]	.06	.59	1.0	4.1	.21	.46	2.2
Pancreas (rat)	13	407	2.4	10.7	2.2	1.2	25.3	21.1	7.4	4.2	16.9	24.4
	10	461	2.2	10.1	2.9	.96	26.0	17.4	8.1	4.7	16.8	20.2
Submaxillary Gland (rat)	13	390	2.6	10.5	2.0	.73	12.6	8.2	3.7	2.7	9.9	17.7
	10	438	2.2	10.5	5.4	.71	11.1	5.8	8.8	2.5	8.3	10.8
Adrenal Gland (human)	1	230	4.4	9.1	1.7	.99	4.0	4.0	9.0	5.8	8.0	23.4

The nuclear protein fell into three classes, related approximately in powers of two. The lowest values were found in the thymus and two of the mouse tumors, ranging from 3.3 to 5.1. The middle range of values was wider (from 7.8 to 15.4) and was found in the majority of normal tissues and tumors. The highest values were found in liver and the human hyperne-

[1] Tissues from individuals were pooled for fractionation and analysis.

[2] Nuclear counts not valid (see text). Values given in the table were calculated on the assumption that the DNA per average nucleus is the same as for other normal and malignant tissues ($10.5 \text{ g} \times 10^{-12}$).

[3] The nuclear RNA values were obtained after correction of the orcinol reading for the color produced by reaction with DNA. In the thymus the concentration of DNA is so high that this correction of DNA is probably meaningless.

phroma (from 19.5 to 26.6). In proportion to cell size, the nuclear protein of thymus was outstandingly high, that of tumors as a group was intermediate, and the remaining values were grouped in the lowest range.

The average cell of both liver and kidney contained very much more mitochondrial protein than did any other cell type included here. However, the concentration of RNA in the mitochondria of liver was considerably

TABLE II

Relation of protein-nitrogen and nucleic acid to average cell weights of various normal tissues.[1]

	DNA	RNA				Protein-N			
		Nucleus	Mitochondria	Microsomes	Supernate	Nucleus	Mitochondria	Microsomes	Supernate
Liver (rat)	1.7	.69	1.9	3.2	1.0	2.9	7.2	4.0	6.7
	1.8	.86	2.0	3.0	1.5	3.7	6.7	3.7	8.1
Liver (mouse)	2.2	.66	2.3	1.7	1.2	3.5	5.4	1.8	6.3
Kidney (rat)	3.1	.45	.42	1.0	1.3	3.2	5.2	3.5	8.1
	3.1	.41	.48	.86	1.0	3.0	4.7	2.8	7.2
Thymus (rat)	30	–	.12	1.6	2.7	10	.45	1.3	5.5
	30	.85	.18	1.6	2.9	11	.61	1.3	6.2
Pancreas (rat)	4.5	.92	.50	11	8.8	3.1	1.8	7.0	10
	4.6	1.3	.44	12	7.9	3.7	2.1	7.6	9.2
Submaxillary Gland (rat)	4.5	.77	.29	4.9	3.2	1.5	1.1	3.9	6.9
	4.6	2.4	.31	4.9	2.5	3.9	1.1	3.6	4.7
Adrenal Gland (human)	2.1	.39	.23	.91	.91	2.0	1.3	1.8	5.3

higher than that found in kidney mitochondria. Tumors were not distinctly different from the majority of normal tissues with respect to the mitochondrial protein or RNA, although the RNA/protein-N ratio was higher than was usually found in normal tissues.

Liver cells contained much more microsomal protein than did any other cell type studied. However, the RNA content of the microsome fraction of pancreas was as high as that of liver, because of the high concentration of RNA in this fraction of pancreas. Kidney cells contained about the same quantity of microsomal protein as did submaxillary gland cells, but the

[1] $g \times 10^{-12}$ constituent/$g \times 10^{-9}$ cell weight.

concentration of RNA relative to protein-nitrogen in this fraction of kidney was low, in sharp contrast to that of the submaxillary gland. In proportion to the size of the average cell, pancreas was outstanding in its high concentra-

TABLE III

Protein-nitrogen and nucleic acid contents of the average cells of various tumors.

	Nuclei per gram $\times 10^6$	Wt. of av. cell ($g \times 10^{-9}$)	DNA, av. nucleus ($g \times 10^{-12}$)	RNA of Av. Cell ($g \times 10^{-12}$)				Protein-N of Av. Cell ($g \times 10^{-12}$)			
				Nucleus	Mitochondria	Microsomes	Supernate	Nucleus	Mitochondria	Microsomes	Supernate
Primary Carcinomas.											
Hepatomas (rat):											
3′-me-DAB	429	2.3	11.2	3.8	1.7	4.6	3.6	14.3	1.9	4.6	12.5
	490	2.0	11.0	4.7	1.4	4.7	4.8	13.5	3.0	4.2	11.4
4′-F-DAB	478	2.1	13.4	4.3	1.6	4.8	3.5	12.8	3.6	4.1	13.1
2-AAF	512	2.0	7.6	2.3	1.9	3.9	3.3	7.8	4.2	3.9	10.8
	402	2.5	11.7	4.2	2.1	4.2	4.0	13.5	3.6	8.0	12.5
Hypernephroma (human)	226	4.3	7.9	4.8	0.93	1.7	2.3	21.8	3.9	5.8	15.0
Transplantable Carcinomas.											
Flexner-Jobling[1] (rat)	474	2.1	11.6	4.3	1.2	1.9	3.6	11.8	2.6	2.8	16.0
	460	2.1	11.6	4.6	1.3	2.7	3.9	13.9	2.7	3.0	13.8
Walker 256 (rat)	455	2.2	11.9	7.5	2.0	5.1	4.2	14.7	1.7	4.6	14.7
	502	2.0	10.5	6.2	0.9	3.9	3.2	15.4	2.3	4.0	13.9
Mammary Carc. (mouse)	743	1.4	9.5	5.2	0.24	2.0	2.8	10.7	0.7	1.5	6.1
Adrenal Carc. (mouse)	725	1.4	9.8	1.5	0.66	3.0	2.2	5.0	2.0	3.2	7.3
	770	1.3	7.1	1.1	1.1	2.3	2.7	5.0	2.2	2.4	7.6
Primary Sarcoma.											
Fibrosarcoma (human)	359	2.8	10.7	5.4	1.5	3.0	4.0	19.4	3.4	4.9	14.1
Transplantable Sarcomas.											
Jensen Sarcoma (rat)	466	2.1	11.3	7.2	0.9	4.8	4.8	13.8	1.7	5.0	14.0
	512	2.0	10.2	7.4	0.9	5.8	5.2	10.8	1.5	4.2	12.8
Lymphosarcoma[1] (mouse)	1 540	0.65	8.3	0.40	0.28	1.5	2.5	5.1	1.0	1.8	5.2

[1] Nuclear counts not valid (see text). Values given for these tumors were calculated on the basis of the DNA per average nucleus obtained on analysis of an ascites suspension of tumor cells.

tion of microsomes. Submaxillary gland, the other exocrine gland included in this study, was second in microsome content, and liver and kidney followed. Tumor cells contained relatively little microsomal material in comparison with the normal cells studied here.

The quantity of RNA which was not sedimentable at 120 000 × g was very high in the cells of pancreas, intermediate in the cells of the other digestive glands studied, and relatively low in the other normal tissues and tumors. In proportion to cell size, the pancreas contained the largest amount of non-sedimentable RNA, but large quantities were also present in the submaxillary gland, thymus, and lymphosarcoma cells. The non-sedimentable protein was closely proportional to cell size, except that the average cell of pancreas contained much more than any other cell type.

The concentration of RNA in terms of protein-nitrogen may be calculated from the data in Tables I and III. Among the normal tissues, the exocrine glands contain unusually high concentrations of RNA in the tissue as a whole, and in each of the cell fractions except for the mitochondria. About 80 per cent of the total RNA of these cells is found in association with the submicroscopic particles and non-sedimentable protein. The tumors as a group also contain relatively high concentrations of RNA in each cell fraction and in the tissue as a whole, but except for the mitochondrial fraction, the RNA concentration of tumors does not exceed that of the exocrine tissues. Kidney is noteworthy for the low concentration of RNA in the cytoplasmic particulates.

The yellow pigment characteristic of the adrenal cortex was found mainly in the microsome fraction. A dark muddy color of the protein precipitates of the mitochondrial fractions was also noted. The protein precipitates of the nuclear and supernatant fluid fractions were almost white. Because of the much greater mass of the adrenal cortex and the greater concentration of the cortical cells, the results of this experiment represent mainly an analysis of adrenal cortical tissue.

It has been convenient in the comparison of tissues to present the results in the form of "biochemical profiles", Figs. 1 and 2. These are arbitrary constructions in which the quantities of protein-nitrogen and RNA in each fraction of the average cell are plotted along the ordinate, while the relative weights of the average cells are represented by the width of the entire profile, in arbitrary units, along the abscissa (designated by the length of the horizontal bar beneath the profile). The vertical bar represents the DNA per average nucleus. The four points constituting the profile are, in order, the analytical values for the nucleus, the mitochondrial, the microsomal, and the final supernatant fluid fractions of the average cell.

If one considers first the protein-nitrogen data, three patterns, or kinds of profile, have appeared. The first, found in rat and mouse liver and rat kidney, is characterized by relatively large quantities of mitochondrial and non-sedimentable protein; the nuclear and microsomal protein components are roughly equal in quantity, and form much smaller fractions than the mitochondria or supernatant protein. Since the nuclear fraction is visibly contaminated with cytoplasmic material, as whole cells and cell fragments, it is probable that the difference between the nucleus and the cytoplasmic protein components within the living cell is even greater.

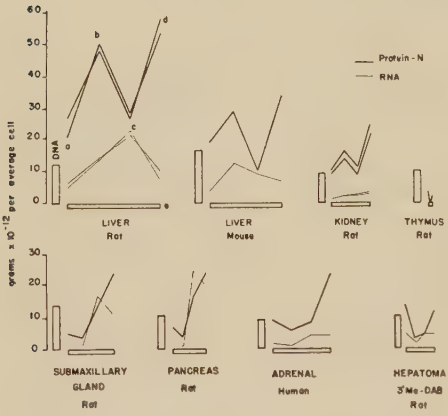

Fig. 1.

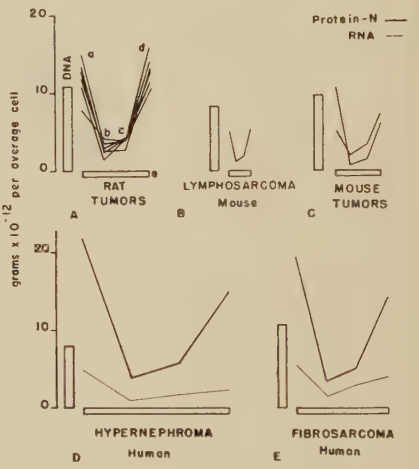

Fig. 2.

Fig. 1. These are arbitrary constructions in which the quantities of protein nitrogen and of RNA in each fraction are represented by the height of each of the four points above the zero line. The first point (a) in each construction represents the nucleus, the second (b) represents the mitochondria, the third (c) the microsomes, and the fourth (d) the final supernatant fluid. The width of the pattern, represented by the horizontal bar (e), corresponds to the weight of the average cell, in arbitrary units. The vertical bar represents the DNA content of the average cell. Three types of pattern are evident: the rat and mouse liver and rat kidney possess an "N" shaped pattern; the rat thymus, human adrenal, and tumor cell possess a U-shaped pattern; and the two exocrine tissues possess a third distinctive pattern.

Fig. 2. The constructions are similar to those of Fig. 1. In A the 6 rat tumor profiles are superimposed; in B and C the three mouse tumors are represented. The apparent difference between the patterns of the two human tumors (D and E) arises almost exclusively from the difference in size of the average cells.

The second pattern is found in the two exocrine glands studied, the pancreas and the submaxillary gland. This type is characterized by a quantity of mitochondrial protein distinctly less than that of the nuclear fraction, in contrast to the pattern characteristic of liver and kidney, while both the microsomal and supernatant fluid protein components are very large,

reaching three to four times the quantity of protein in the nuclear fraction. The pattern of the exocrine glands is distinguished by another fact: the RNA of the microsome fraction is present in larger quantity than the protein-nitrogen, resulting in a crossing of the protein-nitrogen line by the RNA line.

TABLE IV

Relation of Protein-Nitrogen and nucleic acid to average cell weights of various tumors.[1]

	DNA	RNA				Protein-N			
		Nucleus	Mitochondria	Microsomes	Supernate	Nucleus	Mitochondria	Microsomes	Supernate
Primary Carcinomas.									
Hepatomas (rat):									
3′-me-DAB	4.9	1.7	.74	2.0	1.6	6.2	.83	2.0	5.4
	5.5	2.4	.70	2.4	2.4	6.8	1.5	2.1	5.7
4′-F-DAB	6.3	2.0	.76	2.3	1.7	6.1	1.7	2.0	6.2
2-AAF	3.8	1.2	.95	2.0	1.6	3.9	2.1	2.0	5.4
	4.7	1.7	.84	2.1	1.6	5.4	1.4	3.2	5.0
Hypernephroma (human)	2.0	1.1	.22	.40	.53	5.1	.91	1.3	3.5
Transplantable Carcinomas.									
Flexner-Jobling	5.5	2.0	.57	.91	1.7	5.6	1.2	1.3	7.6
	5.5	2.2	.62	1.3	1.9	6.6	1.3	1.4	6.6
Walker 256 (rat)	5.4	3.4	.91	2.3	1.9	6.7	.78	2.1	6.7
	5.3	3.1	.45	2.0	1.6	7.7	1.2	2.0	7.0
Mammary Carc. (mouse)	6.8	3.7	.17	1.4	2.0	7.6	.50	1.1	4.4
Adrenal Carc. (mouse)	7.0	1.1	.47	2.1	1.6	3.5	1.4	2.3	5.2
	5.5	0.95	.85	1.8	2.1	3.8	1.7	1.8	5.8
Primary Sarcoma.									
Fibrosarcoma (human)	3.8	1.9	.54	1.1	1.4	6.9	1.2	1.7	5.0
Transplantable Sarcomas.									
Jensen Sarcoma (rat)	5.4	3.4	.43	2.3	2.3	6.6	.81	2.4	6.7
	5.1	3.7	.45	2.8	2.6	5.4	.75	2.1	6.4
Lymphosarcoma (mouse)	13	.62	.43	2.3	3.8	7.8	1.5	2.8	8.0

[1] $g \times 10^{-12}$ constituent/$g \times 10^{-9}$ cell weight.

The third pattern, found in the adrenal and thymus and in all the tumors, is characterized by the small quantity of the cytoplasmic particulate fractions, and by having proportionately less cytoplasmic protein than the other tissues studied here. This is in agreement with the microscopic appearance of these cells.

The six rat tumor cell profiles are superimposed in Fig. 2A; the RNA lines have been omitted to avoid confusion. It is apparent that the six rat tumors are remarkably similar in the distribution of protein-nitrogen within the cell.

The three mouse tumors are shown in Figs. 2B and 2C. They are similar to each other and to the rat tumor cells in pattern, although differing in size.

The two human tumors are represented in Figs. 2D and 2E. In contrast to the difference in the weights of the average cell in these tumors (represented by the width of the profiles), the amounts of protein-nitrogen and RNA are closely similar in the corresponding cell fractions, as can be seen by comparing the heights of the corresponding points in the two patterns.

With respect to the RNA of the normal tissues, it can be seen that the largest part of the total RNA is found in the microsome fraction of the rat liver and the two exocrine glands, and in the supernate of the kidney and thymus. In the adrenal, the RNA of the microsomes and the supernate are both high. In the tumors, the RNA contents of the nucleus, microsomes and supernate are generally of the same order of magnitude.

DISCUSSION

The advantages of expressing the results of biochemical analyses on a per cell basis, rather than the usual wet or dry weight basis, have been demonstrated in previous work (10, 20). Since most of the connective tissue is removed in the procedure used here, the average cell is essentially an average parenchymal cell. In tissues like the pancreas, with several morphologically distinct types of parenchymal cells, the average cell, however, is of necessity a conglomerate. In spite of this fact, it is felt that expression of results in this form brings us closer to understanding the internal structural and functional relationships of cells, and emphasizes the biological fact that protoplasm functions as cells, and organs, and organisms, but not as a gram of protoplasm, wet or dry.

The application of the same fractionation procedure to such a variety of tissues may be questioned. However, the centrifugal forces used at each step of the procedure are vastly different; on the other hand the size and

density of the particles composing the various cell fractions are very different one from another in any one tissue, but are very similar from one tissue to another. The nuclear and mitochondrial fractions from most of the tissues have been examined with the phase contrast microscope, and the mitochondrial and microsomal fractions of several tissues have been studied with the electron microscope. Except for the nuclear fraction, which always contains those cells which have not been broken during homogenization, the cell fractions examined have been quite pure, showing that in practice as well as theory this fractionation procedure can be applied to a variety of tissues.

The present results indicate, however, that the corresponding fractions of different tissues are not the same. For example, the mitochondrial fraction of liver is different from that of kidney, since the RNA to protein-N ratio of the two is greatly different. Indeed, the kidney mitochondrial fraction as a whole probably corresponds to that mitochondrial subfraction of liver which contains the large mitochondria, with their very low concentration of RNA (12).

The essentially uniform quantity of DNA in the average nucleus of normal and malignant tissues is in complete agreement with the results of other authors (3, 6, 14, 15). However, since these are average values based on analyses of tissues which contain appreciable proportions of polyploid cells (31) they are higher than the values accepted for diploid nuclei (2, 5).

Pancreas, submaxillary gland, and liver, and of course all the rapidly growing tumors, are known to be carrying on relatively intense protein synthesis in contrast to such tissues as adrenal or kidney or thymus. In agreement with the work of the Caspersson (5) and Brachet (4) schools, the RNA content of the average nucleus in the former tissues is higher than in the latter. The results of these workers are in general further corroborated by the high proportion of microsomal RNA in those normal tissues with intense protein synthesis. However, the tumor cells differ from these normal tissues in two respects: the range of nuclear RNA in tumors is distinctly higher than in the normal tissues, and the microsomal and non-sedimentable RNA and protein are generally lower than in these normal tissues, whether considered as absolute or relative quantities in the average cell. Perhaps these differences are more closely related to the presence or absence of rapid cell proliferation than to protein synthesis as such.

The strong basophilia and high ultraviolet absorption (5) observed in histological sections of tumors probably reflect more closely the proportion of RNA to protein than the total RNA in the cell, since components other

than protein and nucleic acids are largely removed by the preparative procedures usually employed. The total RNA in the average tumor cell is in fact rather low, compared with most of the normal tissues studied here. The RNA/protein-nitrogen ratio of tumor cells, however, is very high in all fractions, and tumors are quite comparable in this respect to the exocrine glands, which probably carry on the most intense protein synthesis of any tissue in the body.

Among the tissues included in the present study, the liver and kidney contain unusually large quantities of mitochondrial protein per average cell. Unfortunately, the conclusion that tumors are deficient in mitochondria (22) and those respiratory enzymes known to be associated with mitochondria (27, 28) has been based mainly on comparison of tumors with liver or kidney. The present results indicate that the high mitochondrial protein content of liver and kidney is exceptional, and that tumors are not deficient in mitochondria when compared with such tissues as the exocrine glands, or adrenal, or thymus.

It has been shown by previous workers that tumors more closely resemble one another than do normal tissues, with respect to vitamin content (17), enzyme pattern (7, 8, 18), and nucleic acid content (28). Nevertheless it is surprising to find agreement of such precision among the results of the tumor analyses in the present study. These results suggest that tumor cells are as distinctive as liver or pancreas cells in the protein and nucleic acids of the various cell organelles. This conclusion would not be in disagreement with the experience of the pathologist, that tumors are usually readily differentiated on microscopic and often on gross examination. The connective tissue of the tumors in the present study has been removed to a large extent by straining the tissue through a plastic mincer before homogenization. The individual cells of the varieties of tumor studied here are very similar when observed in a homogenate. The pathologist's diagnosis rests largely on differences in the architectural pattern of the tumor tissue, which in turn are largely an expression of the connective tissue element, as is the gross appearance of a tumor.

The unusually high concentration of non-sedimentable protein in the average cell of pancreas may represent digestive enzymes awaiting secretion.

Nuclear protein classes within single tissues have been demonstrated by spectrophotometric measurements of individual nuclei (1, 13) and by determination of the protein content of average nuclei (11). The present results suggest that the nuclear protein characteristic of different tissues might also occur in classes corresponding to the powers of two.

In conclusion, the possibility might be suggested that mitochondria and microsomes are related, in quantity and diversity of kind, to the degree of functional specialization of the cell; and that liver, kidney, and exocrine gland cells are more highly specialized, in keeping with the greater functional diversity which seems to be characteristic of them in comparison with adrenal or thymus or tumor cells.

SUMMARY AND CONCLUSIONS

A variety of normal and malignant tissues, obtained mainly from the rat, has been fractionated by a modification of the method of Hogeboom *et al.* (9).

Comparison of these tissues has allowed them to be grouped into three classes on the basis of the relative proportions of the cytoplasmic particulates. Liver and kidney cells are distinguished by a relatively high mitochondrial content, in terms of protein-nitrogen. The two exocrine glands studied, pancreas and submaxillary gland, are characterized by a very high proportion of microsomal material, but a rather low proportion of mitochondria. About 80 per cent of the RNA of the latter cells is found in association with the submicroscopic particles and non-sedimentable proteins. Thymus, adrenal, and all the tumors studied are characterized by relatively low proportions of the cytoplasmic particulate fractions, and by a relatively small quantity of cytoplasmic protein as a whole.

Comparison of the tumors among themselves showed that they were remarkably similar in the amounts of protein and RNA in corresponding cell fractions, the rat tumor cell values differing little more than would the results of successive fractionations of a normal tissue.

The liver tumors were supplied through the courtesy of Drs. E. C. and J. A. Miller of this laboratory; the human tissues were obtained through the courtesy of Dr. Walter Jaeschke, surgical pathologist of the State of Wisconsin General Hospital.

I wish to express my gratitude to Dr. Hans Ris for his advice and encouragement.

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Experimental Cell Research, 6, 45–55 (1954)

ULTRAVIOLET INACTIVATION OF *SACCHAROMYCES* DURING THE BUDDING CYCLE[1]

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Received April 18, 1953

Cellular susceptibility to ultraviolet radiation is governed primarily by the sensitivity of the nucleus (7, 11). Carlson's extensive studies of mitotic grasshopper neuroblasts have shown the existence of differential nuclear sensitivity at various mitotic stages in terms of the disruption of particular nuclear organelles and the inhibition of karyokinesis (2, 3, 4). Virtually nothing is known, however, of the fluctuations in the kinetics of ultraviolet inactivation of cells during the mitotic cycle. Attempts at such determinations are handicapped ordinarily by the fact that complex organisms in which stages of mitosis are most clearly defined are not amenable to survival curve analyses; the principal difficulties arise from the absence of simultaneous phases of division occurring naturally in control populations of cells and the inadequacy of criteria for determining the destruction of individual cells.

In this study of ultraviolet inactivation of budding yeasts, it has been possible to circumvent both these difficulties and to establish an accurate correlation by survival curve comparisons between cellular sensitivity to radiation and the known cytologic and metabolic developments of the budding cycle. It is shown that (a) the rate of logarithmic inactivation of a suspension of monokaryotic cells is related directly to the number of events required to inactivate the individual cells comprising the suspension. Both the inactivation rate and the number of events involved in inactivation depend upon the cellular desoxyribose nucleic acid content. (b) The number of events required to inactivate dikaryotic cells is governed by the total cellular desoxyribose nucleic acid content. The rate of logarithmic inactivation for such individuals, however, is affected by the distribution of desoxyribose nucleic acid between the two nuclei. (c) An increase in the proportion of cytoplasm to desoxyribose nucleic acid increases the amount of damage cells may sustain prior to inactivation but does not influence the

[1] This work was supported by research grants from the U. S. Atomic Energy Commission, Contract AT(11-1)-176, and the Office of Naval Research, Department of the Navy, Contract (NR 164-140).

rate of logarithmic inactivation. (d) Inactivation does not result from lethal gene mutation. The events leading to inactivation appear to involve the nuclear heterochromatin.

MATERIALS AND METHODS

Fresh baker's yeast cake is composed almost exclusively of single cells. When transferred to nutrient broth and aerated, these cells complete their first budding cycle in excellent synchrony. The occurrence of parallel nuclear changes in all cells of such populations has been confirmed through nucleic acid analyses (12).

For these experiments cells from a cake of baker's yeast showing less than 0.5 per cent budding were washed once with M/15 monopotassium phosphate and suspended to a density of approximately 10^7 cells/ml in 100 ml of Burkholder's minimal medium (14), less asparagine, in a one liter flask. This growth suspension was incubated on a reciprocal shaker at room temperature, 28–30°C.

A non-budding control sample was removed for ultraviolet survival curve determination after 5–10 minutes incubation. This brief incubation period permits the cells to break dormancy and to establish normal metabolic conditions prior to budding. Subsequent samples were removed for irradiation after 42–63 minutes incubation, at which time all viable cells possess minute buds, and after 63–75 minutes, 75–86 minutes, 86–114 minutes or 120–150 minutes incubation, a short while before the onset of the second, asynochronous budding cycle.[1] Over the course of these experiments, it was necessary to sample within time ranges since cells from individual yeast cakes were not in identical physiologic states and therefore exhibited slightly different budding rates.

Yeast cells removed from the growth flask were washed twice in M/15 monopotassium phosphate and suspended in M/15 monopotassium phosphate to a concentration of 4.5×10^2 cells per ml. Thirty ml of the suspension was transferred to an open petri dish and irradiated at a distance of 52 cm from a Hanovia quartz envelope ultraviolet lamp delivering 95 per cent of its ultraviolet energy in wave length 2537 Å. During irradiation the suspensions were agitated by means of a magnetic stirrer. Duplicate one-half milliliter samples were removed at intervals during irradiation (Fig. 1) and plated directly, without dilution, in the following medium: dextrose, 2 per cent ammonium sulfate 0.1 per cent, Budweiser yeast extract solids, 0.2 per cent, monopotassium phosphate, 0.15 per cent, magnesium sulfate, 0.5 per cent, and agar, 2 per cent. Plates were incubated in the dark for 3 days at 30°C and survival was determined by average macroscopic colony counts taken with the aid of a Quebec colony counter.

Microscopic examinations of heavy suspensions of budding cells carried through the entire experimental procedure gave assurance that all buds

[1] Methylene blue staining of the yeast cakes employed in this study indicated 85 per cent to 90 per cent viability. This checked directly with the percentage of budding cells in the growth suspensions.

remain attached to their mother cell from the time samples were removed from the growth flask through the time of plating.

Survival curves were plotted on semi-logarithmic paper with S, the fraction surviving, plotted along the axis of ordinates and the dose D, measured in seconds of exposure on the axis of abscissae.

The points of the curves shown in Fig. 1 are average survival values for the corresponding dosages obtained from a number of independent determinations. All survival curves are sigmoid and show a threshold or shoulder phase which leads into the phase of logarithmic inactivation at approximately 40 per cent survival. The threshold portions of the curves were fitted to the experimental points by eye. The straight line portion of each curve ($S \geqq 40$ per cent) was fitted by the method of least squares to the equation: $\ln S = \ln n - KD$, where S is the fraction surviving, n is the intercept of the straight line with the axis of ordinates, K is the slope of the line and D is the dose measured in seconds of exposure. According to the multi-target theory, the intercept, n, represents the number of radiation-sensitive sites, or targets, which must be inactivated to prevent cell division (1). Inactivation kinetics are discussed in terms of the multi-target theory rather than the multi-hit theory since yeast survival curves correspond much more closely in form to the multi-target model (15).

RESULTS

Ultraviolet survival curves for yeast populations at successive stages of budding are presented in Fig. 1. Standard deviations for all points of each survival curve are given in Table I. Curve A is the survival curve for unbudded cells.

With the inception of budding a significant rise in sensitivity to ultraviolet is reflected in the shortening of the threshold period prior to the phase of logarithmic inactivation and a marked increase in the rate of logarithmic inactivation (Curve B). At this time, the cellular target value rises from 40 to 77. As budding continues, however, the radiation sensitivity of mother cell-bud complexes decreases decidedly (Curves C and D). Through the mid-portion of the budding cycle their target values and rates of logarithmic inactivation remain approximately constant and well below those for control cells. A continuing development of resistance is indicated only by the progressively larger threshold dosage sustained preceding logarithmic inactivation. Toward the end of the budding cycle, cellular rates of logarithmic inactivation and target values begin to rise. At the cycle's completion, mother

cell-bud complexes withstand a maximal threshold dose but show greatly increased sensitivity during the phase of logarithmic inactivation (Curve F). Their survival curve yields the same slope and approximately twice the target value as that for unbudded control cells.

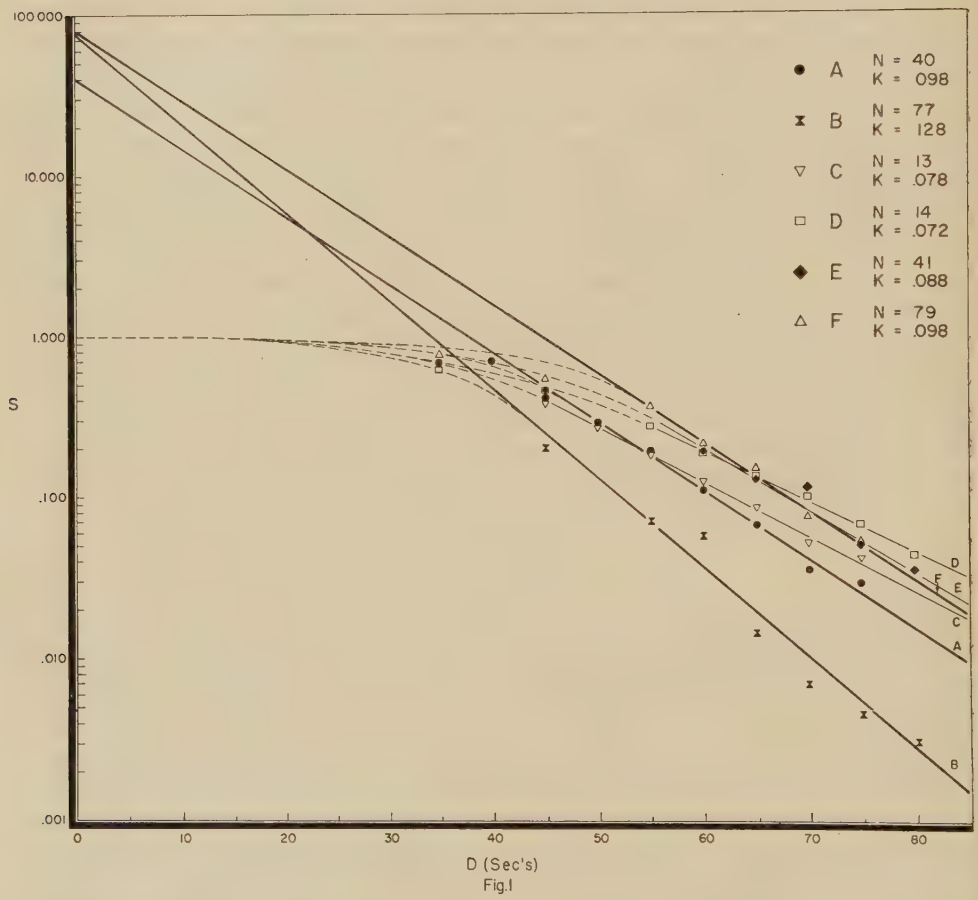

Fig. 1. Curves A through F are survival curves for cell populations at successive stages of a single budding cycle. The target value, n, and rate of logarithmic inactivation, K, for each curve are given in the legend.

DISCUSSION

Mitosis and budding. Genetical studies of *Saccharomyces* have established the equational partitioning of nuclear material at yeast cell division. Unfor-

tunately, the mitotic stages during the budding process are poorly determined by direct observation of chromosome activity since these structures are minute and exceedingly difficult to differentiate. However, the major portion of the yeast cell chromatin is localized as a conspicuous heterochromatic structure, the centrochromatin, at the periphery of the nuclear vacuole (9). Since the euchromatic portion of the chromosomes must remain associated with the centrochromatin, the behavior of the readily discernable heterochromatin serves as a satisfactory index of karyokinetic development.

At the onset of budding the centrochromatin body remains immobile and in joint contact with the nuclear vacuole and centrosome. Nucleic acid analyses have shown that during or immediately preceding this period the desoxyribose nucleic acid (DNA) content of the cell doubles (12). Accumulation of desoxyribonucleotides is a characteristic feature of the formation of mitotic chromosomes and may be taken here as indicative of a prophase.

Toward the mid budding period, the heterochromatin migrates to the mother cell-bud juncture and occupies a position suggesting a metaphase

TABLE I

Numerical values and standard deviations for all survival points shown in Fig. 1. Each value is the average for the number of independent determinations indicated.

No. of runs per curve	Dose in seconds of exposure									
	35	45	50	55	60	65	70	75	80	
A 16	68.2	40.9	29.0	19.2	11.0	6.6	3.5	2.8		Aver. % Survival
	6.6	9.0	9.5	7.3	5.4	3.0	3.4	1.1		Stand. Dev.
B 4		20.3		7.2	5.6	1.5	0.7	0.5	0.31	Aver. % Survival
		4.9		1.4	0.3	0.2	0.3	0.10	0.06	Stand. Dev.
C 3	67.7	38.2	27.2	18.7	12.4	8.5	5.2	4.2		Aver. % Survival
	4.2	1.6	4.1	2.0	1.3	1.5	1.4	0.2		Stand. Dev.
D 6	61.9	45.8		27.7	18.9	13.6	10.1	6.8	4.4	Aver. % Survival
	[1]	7.0		8.3	5.4	4.9	1.6	0.9	0.5	Stand. Dev.
E 6		45.7		27.9		14.3	11.5	5.4	3.00	Aver. % Survival
		8.2		9.1		5.1	3.7	1.7	1.6	Stand. Dev.
F 8	77.4	54.0		36.7	21.60	15.0	7.6	5.4		Aver. % Survival
	6.0	15.7		14.9	13.7	9.6	3.9	2.3		Stand. Dev.

[1] A single survival determination made at this dosage.

alignment of chromatin. Anaphase is observed during the latter stages of budding when a portion of the centrochromatin material extends from the mother cell into the bud. Telophase follows with splitting of the heterochromatic mass and its equal distribution between mother cell and bud. Within a brief interval the interphase nuclei are reestablished and the mother cell-bud complex is capable of initiating further buddings.

Effect of cytoplasm upon threshold sensitivity. The total response of cells to damage scored upon the nucleus by ultraviolet radiation is conditioned profoundly by the existent ratio of cytoplasm to nucleoplasm; increased concentrations of cytoplasm favor nuclear repair and cellular recovery (10). For this reason a direct correlation between cellular susceptibility to radiation and the constitution of the mitotic nucleus is precluded by the peculiar cytoplasmic activity of dividing yeasts.

Among most organisms nuclear division is accompanied by a state of cytoplasmic quiescence. Cellular activity is channeled wholly toward synthesis of nuclear proteins and nucleotides and the provision of energy required for karyokinetic movement. Cessation of growth is associated with distribution of nucleolar material to the mitotic chromosomes and a concomitant diminution of the concentration of cytoplasmic ribonucleotides (5, 8). During the yeast cell budding cycle, however, maximum synthesis of cytoplasmic materials is concurrent with mitosis. Protein metabolism does not appear to be related to the nucleolar heterochromatin but to ribose-nucleic acid rich cytoplasmic organelles which maintain their integrity throughout the cell's life cycle (6). Thus from interphase to the time for telophase reconstitution of functional nuclei, there exists a continually developing gradient in cellular cytoplasmic content.

The family of curves shown in Fig. 1 illustrates the influence of cytoplasmic growth in expanding cellular capacity for withstanding radiation inactivation. The marked sensitivity of prophase cells (Curve B) is attributable to the fact that the slight cytoplasmic increase at this stage is insufficient to compensate for the doubling of nuclear material. Throughout the remainder of the cycle, however, as the DNA concentration remains constant and the cytoplasmic volume increases, a consistent rise is observed in the threshold dosage required to initiate inactivation. The increasing rates of logarithmic inactivation toward the termination of the budding cycle (Curves E and F) indicate that cellular sensitivity during the phase of logarithmic inactivation is not related critically to the cytoplasm/nucleoplasm ratio. Substantial proof that the cytoplasmic effect is restricted to threshold sensitivity is provided by comparison of survival curves C and D for cell populations at

successive stages of the mid budding cycle. An average growth interval of only ten minutes elapsed between these survival determinations. Since, under the conditions of these experiments, the completion of a single budding cycle required from 2 to 2.5 hours, it can be assumed that merely a slight cytoplasmic increase distinguished population D from C. The target values and inactivation rates for both populations are virtually identical. The influence of the cytoplasmic difference is reflected significantly only in the threshold portions of the curves. It is inferred that the cytoplasm/nucleoplasm ratio determines the extent of damage cells may sustain prior to inactivation, but exerts no influence upon the final rate at which they are inactivated; therefore, the resistance afforded by increase in cytoplasm does not result from mechanical screening of potentially effective radiations by cytoplasmic proteins and ribonucleotides, but represents an enhanced cellular capacity for repairing damage inflicted by threshold doses of radiation.

Association of rates of logarithmic inactivation and target values. Throughout the budding cycle increasing rates of logarithmic inactivation are associated with higher target values (Fig. 2). A study of ultraviolet inactivation of polyploid *Saccharomyces* revealed the same relation; cells of higher degrees

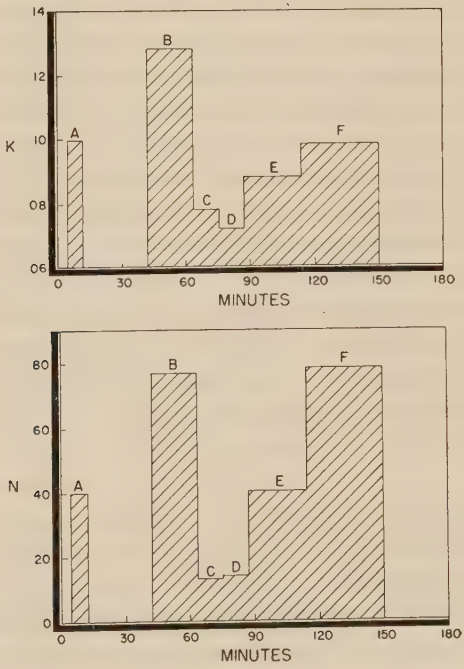

Fig. 2. Histograms showing the general correlation of the variations in cellular rates of logarithmic inactivation and target values over the course of a single budding cycle.

of polyploidy yield both greater n and k values (15). Comparison of the results of that study with the survival curves obtained here permits a correlation of these survival curve constants with the cellular DNA content.

Diploid yeasts contain twice as much DNA as haploids (13) and prophase cells have twice the DNA content of interphase cells. The average rate of logarithmic inactivation for a number of haploid strains was 0.041 and that for diploid strains, 0.051. Baker's yeast cell inactivation rates rise from a control interphase value of 0.098 to a prophase value of 0.128. The similarity in the ratios of the inactivation rates for haploid to diploid and interphase to prophase cells, 0.80 and 0.77 respectively, implies that DNA concentration determines cellular sensitivity to ultraviolet radiation at least during the phase of logarithmic inactivation. Doubling of target values concomitant with the duplication of chromatin in prophase cells is decisive evidence that ultraviolet absorption by desoxyribonucleotides is the primary process initiating inactivation and that as DNA increases, the number of radiation events required for inactivation and the rate at which they occur also increase. This generalization must be partially qualified, however, for though the total quantity of DNA per cell remains constant from prophase through the completion of budding, target values and inactivation rates fall considerably during the mid budding cycle. Since the cytoplasm/nucleoplasm ratio does not enter into the determination of n and k values, it is inferred that the concentration of effectually absorptive DNA varies with mitosis. It is particularly interesting that the minimum DNA concentration effective in inactivation exists at metaphase (Curves C and D)—the stage reported by Carlson and Hollaender as most resistant to ultraviolet retardation of karyokinesis (3).

The significance of the target value. Though the association of target values with DNA activity indicates the correctness of the multi-target theory, aspects of the experimental results suggest a reconsideration of certain fundamental assumptions implicit in the theory.

The multi-target hypothesis presumes the existence of a number of radiation-sensitive sites within the cell, each of which is inactivated independently, but with equal probability, by absorption of a single effective quantum according to the equation:

$$S_t = e^{-KD} \tag{1}$$

where K is the rate of target inactivation, D is the dose, and S_t the probability of target survival.

If n number of targets exists and all must be inactivated to render the cell inviable, then cell survival, S_c, is given by the equation:

$$S_c = 1 - (1 - e^{-KD})^n \quad (2)$$

For sufficiently large volumes of D, terms in the binomial expansion $(1 - e^{-KD})^n$ of powers higher than e^{-KD} may be ignored and the equation

$$\ln S_c = \ln n - KD \quad (3)$$

is obtained. Plotting this equation produces a straight line on semi log paper crossing the S axis at $D = 0$, $S = n$. Thus extrapolation of the straight line portion of an experimental to zero dose, gives the target number.

For increasing values of n, the theoretical curves of equation 2 yield a family of curves of identical slopes with increasing threshold phases. Since cellular inactivation requires the destruction of all targets, the threshold periods represent the time required to hit $n - 1$ targets. Experimental survival curves A and F for unbudded control cells and dikaryotic mother cell-bud complexes conform well to the theoretical expectations. The mother cell-bud curve shows twice the target value, the same slope and a greater threshold phase than that for control cells. However, target duplication in prophase cells (Curve B) is accompanied by an unpredicted reduction of the threshold period and a sharp increase in the rate of logarithmic inactivation. This observation could, of course, be reconciled with the theory by assuming enlargement of the effective target cross section at prophase. Since the K value measures the inactivation rate of a single target, the decreased threshold would follow from the increased rate of logarithmic inactivation. Such argument, however, is not permissible, for previous comparison of survival curves C and D has established that the threshold period is related primarily to the cytoplasm/nucleoplasm ratio and varies independently of K and n values. It can be concluded that radiation damage is not accumulated as theoretically proposed and that either the inactivation rate for the last, n^{th}, target is different from that for the preceding $n - 1$ targets or that not all n targets are inactivated independently. The latter possibility is strengthened particularly by the observation that the inactivation rate for cells possessing a given number of targets is greater when all targets are associated with a single nucleus (Fig. 1, Curve B) than when they are equally partitioned between two discrete nuclei (Fig. 1, Curve F).

While discrepancies between the experimental results and certain postulates involved in the derivation of the target notation demand re-evaluation of the manner in which effective radiation damage is scored, they do not necessarily obviate the possibility that the target value has a real biological reference. In fact, the correlation between empirically determined n values and cellular

DNA concentrations substantially supports the contention that the target number is a meaningful measure of primary processes participating in cellular inactivation.

The nature of the inactivation processes. Implication of DNA as the critical ultraviolet absorbing material signifies a genetic basis for radiation inactivation. However, neither of the two cardinal genetic disturbances induced by ultraviolet radiation—chromatid breakage or recessive gene mutation—can account satisfactorily for the kinetics of cellular inactivation.

Swanson's investigations of the effects of ultraviolet upon prophase *Tradescantia* pollen have shown that chromatid breakage is materially suppressed at this stage (16). If this finding is taken to represent a general case, then the increased yeast cell sensitivity at prophase eliminates chromatid aberration as a significant aspect of inactivation. On the other hand, inactivation through recessive lethal mutation requires the destruction of all alleles at any given locus. Nucleic acid determinations (Ogur, personal communication) and analyses of ultraviolet and X-ray survival curves (Sarachek and Lucke, unpublished) indicate that baker's yeast cells are triploid. Considering that each chromosome set must contain several hundred (m) genes, the target values for non-budding control cells should approximate $(3)^m$, a number far in excess of the 40 experimentally obtained.

Exclusion of chromatid and genic damage as causes of ultraviolet inactivation does not in itself eliminate the possibility that destructive absorption occurs at the euchromatic chromosomal DNA. In the case of the yeast cell, however, these data together with the peculiar preponderance of DNA at the nongenic, centrochromatin suggests that the nuclear heterochromatin serves as the critical absorbing material. Since the yeast cell heterochromatin is associated at all times with both the chromosomal euchromatin and the centrosome, inhibition of cell division could arise either through disturbances of the coordinated functioning of the entire gene complement or anomalous partitioning of nuclear material at mitosis.

SUMMARY

Analysis of the survival curves of cells of a synchronously budding yeast population exposed to ultraviolet reactivation has led to the following conclusions:

1. The rate at which monokaryotic cells in a population are inactivated is associated directly with the number of events required to inactivate the individual cells. In general, increases in cell target values are accompanied

by increases in rates of logarithmic inactivation. Both survival curve constants are related to the cellular desoxyribose nucleic acid content.

2. Target values for dikaryotic cells are related to the total cellular desoxyribose nucleic acid content. The rates of logarithmic inactivation of dikaryons are conditioned by the distribution of desoxyribose nucleic acid between two nuclei as well as the total cellular desoxyribose nucleic acid content.

3. Variations in the relative amounts of cytoplasm and desoxyribose nucleic acid affect the amount of damage cells may sustain prior to inactivation but have no discernable effect upon cellular inactivation rates or target values.

4. The primary events in the inactivation processes are nongenic and probably involve the heterochromatin.

The author gratefully acknowledges the assistance of Mr. Ernest Shult in performing the statistical computations. He also wishes to thank Dr. Carl C. Lindegren for valuable advice during the preparation of this manuscript.

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PHYSIOLOGICAL STUDIES ON FERTILIZATION AND ACTIVATION OF FISH EGGS

V. THE ROLE OF CALCIUM IONS IN ACTIVATION OF *ORYZIAS* EGGS

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Received April 18, 1953

It has been shown that the ripe, unfertilized eggs of the medaka (*Oryzias latipes*) can be kept in a fertilizable condition for a number of hours in isotonic Ringer's solution, while they lose the capacity for fertilization and activation within a short period after being immersed in fresh water (14). Fertilization and development take place in the isotonic (M/7.5) Ringer's solution. Hence *Oryzias* eggs furnish an excellent material for the analysis of fertilization and activation processes in the teleostean eggs.

As has been shown previously (14, 16), numerous cortical alveoli (10–40 μ in diameter) are embedded in the cortical layer of the unfertilized eggs of the medaka. Upon fertilization the breakdown of the cortical alveoli takes place in a wave-like manner, beginning near the animal pole where the spermatozoon enters the egg and ending at the vegetal pole. Following this change the separation of the egg membrane takes place. Essentially similar cortical changes can be provoked by a number of activating agents.

It has also been shown that when the unfertilized eggs are washed several times with Ca-free Ringer's solution or immersed in the isotonic solution of sodium oxalate (M/11) for fifteen minutes, they reversibly lose the capacity for fertilization. These eggs recover their capacity for fertilization when they are transferred to Ringer's solution. Magnesium ions can be substituted for calcium ions, but the former require several times the concentration of the latter. The spermatozoa are very active in Ca-free Ringer's solution. These facts are quite in harmony with the experiments of Loeb (8, 9) on sea urchin eggs. Moser (11, 12) has shown that calcium ions are necessary at the activation of sea urchin (*Arbacia*) eggs with chemical (saponin and toluene) and physical agents (pricking, ultraviolet radiation and direct current). Heilbrunn (cf. 1) has formulated a general theory of stimulation in which Ca release from the cortical protoplasm and the subsequent combination of these calcium ions with the inner protoplasm are regarded as fundamental phenomena in stimulation. In view of the importance of calcium ions in stimulation

and excitation, a closer analysis of activation in Ca-free media or in media which precipitate or inactivate Ca ions has been attempted along the following lines.

MATERIAL AND METHODS

The ripe, unfertilized eggs of the orange-red type of the medaka (*Oryzias latipes*) were used as material. Egg-loadened females were isolated from the males the day before the experiments were carried out. The ripe unfertilized eggs were obtained by dissecting and isolating the ovary from the fish, since the stripping method usually injures some eggs, resulting in activation from mechanical injury. The excised ovaries were placed in isotonic Ringer's solution (15), and the ripe ova were isolated with glass needles. One can easily distinguish the ripe ova from the unripe ones by their size and transparency. In the Ringer's solution, the majority of the eggs remains in the normal condition for a number of hours and can be fertilized when inseminated in solution. A few (5-6 per cent) of the eggs were activated in the solution. These eggs were discarded. Autoactivation, however, takes place within five minutes after isolation from the ovary. Afterwards no eggs show autoactivation in the Ringer's solution. These unfertilized eggs were used as material in experiments on activation with activating agents. In pricking experiments, the egg was held tightly in a glass loop. The isotonic Ringer's solution (M/7.5) used has the following composition M/7.5 NaCl 100 parts + M/7.5 KCl 2.0 parts + M/11 $CaCl_2$ 2.1 parts, pH adjusted to 7.3 by adding a small amount of N/10 $NaHCO_3$ (15).

RESULTS

The role of calcium ions in autoactivation. It has been shown in a previous paper (14) that a majority of the ripe unfertilized eggs remained unchanged in the isotonic Ringer's solution. Only a few (6 per cent) eggs undergo autoactivation in this medium. In order to determine whether calcium ions play a role in autoactivation, the following experiments were carried out. The ripe unfertilized eggs were isolated from the ovary and were immediately immersed in the following isotonic solutions: Ringer (control), Ca-free Ringer, citrated Ringer, sodium citrate solution, oxalated Ringer and sodium oxalate solution. The results are shown in Table I. It will be seen that the percentage of autoactivation decreases remarkably in Ca-free Ringer's solution and other media which remove free calcium ions. No eggs show autoactivation in oxalated Ringer or in a solution of sodium oxalate. It may be safe to conclude that autoactivation is inhibited in Ca-free media. In other words, calcium ions are necessary in autoactivation.

Activation with pricking in Ca-free media. It has been shown in a previous paper that when ripe, unfertilized eggs are washed several times with Ca-free

Ringer or treated with an isotonic solution of sodium oxalate for fifteen minutes, the eggs lose the capacity for fertilization and activation with pricking. Experiments on activation with pricking in Ca-free Ringer and in oxalate solution were repeated quantitatively.

TABLE I

Auto-activation of unfertilized eggs in various media. Ovaries were dissected out and the ripe ova were immersed in the solutions.

Media	Composition of media			No. of obs.	Total number of eggs	Number of autoactivated eggs	Per cent of autoactivation
Isotonic Ringer	M/7.5	NaCl	100	10	260	13	5.0
	M/7.5	KCl	2.0				
	M/11	$CaCl_2$	2.1				
Isotonic Ca-free Ringer	M/7.5	NaCl	100	10	184	2	1.1
	M/7.5	KCl	2.0				
Isotonic citrated Ringer	M/7.5	NaCl	100	5	114	1	0.9
	M/7.5	KCl	2.0				
	M/15	Na-oxalate	5.0				
Isotonic Na-citrate	M/15	Na-citrate		10	139	1	0.7
Isotonic Oxalated Ringer	M/7.5	NaCl	100	5	57	0	0
	M/7.5	KCl	2.0				
	M/11	Na-oxalate	4.0				
Isotonic Na-oxalate	M/11	Na-oxalate		10	182	0	0

The ripe unfertilized eggs were washed several times (8 times) with M/7.5 Ca-free Ringer for various periods and then pricked with a glass needle whose tip was 15 μ in diameter. The results are shown in Table II. After immersion for thirty minutes in Ca-free Ringer's solution only 24 per cent of the eggs showed activation. If the pricked eggs are put back into Ringer's solution at ten minutes after pricking, 86 per cent of them undergo activation without further pricking. It follows that 62 per cent of the eggs failed to show breakdown of the cortical alveoli in Ca-free Ringer and undergo activation on return to Ca-containing medium. We are led to conclude that something happens in Ca-free Ringer in these eggs, but the breakdown of the cortical alveoli is inhibited in this medium.

A number of experiments were done in which calcium was removed from the medium, and possibly from the egg cortex, by means of oxalate. When the unfertilized eggs are immersed in an isotonic solution of sodium oxalate (M/11) for 10–15 minutes, none of the eggs show visible cortical changes on stimulation with pricking at the animal pole (Table III). If these eggs are transferred to Ringer's solution at ten minutes after pricking, 62–65 per cent of them undergo typical cortical changes without further pricking. The fact that the oxalate solution itself is not an activating agent was shown by a number of experiments, in which normal eggs were immersed in oxalate solution for various periods and then transferred to Ringer's solution. No eggs showed activation.

TABLE II

Ripe, unfertilized eggs were washed several times (8 times) with M/7.5 Ca-free Ringer's solution and then pricked at the animal pole with a 15 μ needle in the solution. Temperature 27°–31° C.

Time in Ca-free Ringer (Min.)	No. of obs.	In Ca-free Ringer			10 min. after prickning → Ringer		
		Total number of eggs	No. of activated eggs	Per cent of activation	Total number of eggs	No. of activated eggs	Per cent of activation
0 (control)	5	30	30	100	30	30	100
5	5	31	20	65	31	28	90
15	5	26	13	50	26	24	92
30	5	29	7	24	29	25	86

TABLE III

Ripe, unfertilized eggs were immersed in M/11 Na-oxalate solution and were pricked at the animal pole with 15 μ needle in the solution. Ten minutes after pricking they were returned to Ringer's solution. Temperature 28° C.

Time in oxalate solution (Min.)	No. of obs.	In oxalate solution			10 min. after prickning → Ringer's solution		
		Total number of eggs	Number of activated eggs	Per cent of activation	Total number of eggs	Number of activated eggs	Per cent of activation
0 (control)	6	32	32	100	32	32	100
5	6	31	1	3	31	20	65
10	6	31	0	0	31	20	65
15	6	33	0	0	33	21	62

From these experiments it may be safe to conclude that something happens in Ca-free media on stimulation with pricking but the cortical change is inhibited in the oxalate solution.

TABLE IV

Ripe unfertilized eggs were treated with 1 per cent sodium oleate-M/10 Ca-free Ringer's solution and then transferred to M/11 Na-oxalate solution. After five minutes they were returned to Ringer's solution. Eggs showing activation followed by cytolysis are marked with an asterisk. Temperature 29°–32° C.

Time of exposure in min.	No. of obs.	In oxalate solution			In Ringer's solution		
		Total number of eggs	Number of activated eggs	Per cent of activated	Total number of eggs	Number of activated eggs	Per cent of activated
0 (control)	5	30	0	0	30	0	0
1	5	30	0	0	30	7	23
2	5	30	0	0	30	17	57
4	5	29	0	0	29	21	72
8	5	28	7*	25*	28	25*	89*

Chemical activation in Ca-free media. It has been shown in a previous paper (16) that a solution of sodium oleate causes the activation of unfertilized eggs. Since this agent is a calcium precipitant, it was necessary to dissolve it in a Ca-free Ringer's solution. The solution used was 1 per cent solution of sodium oleate (Merck) in M/10 Ca-free Ringer's solution. This solution is osmotically equivalent to M/7.5 Ringer's solution. The ripe, unfertilized eggs were treated with this solution for 1–8 minutes. The eggs showed no cortical change in the solution during this period. When they were transferred to Ringer's solution, they showed typical cortical changes.

In order to determine whether or not these cortical changes will take place in an oxalate solution, the unfertilized eggs treated with 1 per cent solution of sodium oleate-M/10 Ca-free Ringer's solution for 1–8 minutes were transferred to isotonic (M/11) sodium oxalate solution. The results are shown in Table IV. No eggs showed the cortical changes when the time of exposure was 1–4 minutes. These eggs showed typical cortical response when they were transferred to Ringer's solution. Some eggs treated with the oleate solution for 8 minutes showed cortical response when transferred to oxalate solution. But these eggs soon undergo cytolysis. Cytolyzed eggs are marked with an asterisk in the table.

In a previous paper (16) it has been shown that when unfertilized eggs are

treated with 0.1 per cent saponin solution in Ringer or in Ca-free Ringer for two minutes, all eggs showed activation. In order to see whether or not saponin in citrate or oxalate solution induces activation, the following experiments have been carried out. Ripe unfertilized eggs were washed with Ca-free Ringer for 15 minutes and then treated with 0.1 per cent saponin in isotonic sodium citrate (M/15) or in isotonic sodium oxalate (M/11) for two minutes and finally transferred to Ca-free Ringer's solution. As is shown in Table V, eggs treated with 0.1 per cent saponin-M/15 citrate showed no activation, while 28 per cent of the eggs treated with 0.1 per cent saponin oxalate showed activation. Saponin-Ringer induces activation in all eggs (100 per cent).

In another series of experiments, ripe unfertilized eggs were washed with Ca-free Ringer for 15 minutes, treated with 0.1 per cent saponin-M/11 sodium oxalate for two minutes, and then transferred to Ringer's solution. The results are shown in Table VI. It will be seen that a high percentage of eggs showed typical activation.

TABLE V

Ripe unfertilized eggs were washed with Ca-free Ringer for 15 minutes, exposed to 0.1 per cent saponin-M/15 Na-citrate or 0.1 per cent saponin-M/11 Na-oxalate for 2 minutes, and then transferred to Ca-free Ringer's solution. Temperature 24°–26° C.

Solutions	No. of obs.	Total number of eggs	Number of activated eggs	Per cent of activation
0.1 per cent saponin-M/15 Na-citrate	5	29	0	0
0.1 per cent saponin-M/11 Na-oxalate	5	29	8	28
0.1 per cent saponin-M/7.5 Ringer . .	5	29	29	100

TABLE VI

Ripe unfertilized eggs were washed with Ca-free Ringer for 15 minutes, exposed to 0.1 per cent saponin-M/15 Na-citrate or 0.1 per cent saponin-M/11 Na-oxalate for 2 minutes, and then transferred to Ringer's solution. Temperature 24°–26° C.

Solutions	No. of obs.	Total number of eggs	Number of activated eggs	Per cent of activation
0.1 per cent saponin-M/15 Na-citrate	5	30	24	80
0.1 per cent saponin-M/11 Na-oxalate	5	30	28	93
0.1 per cent saponin-M/7.5 Ringer . .	5	30	29	97

Ca release from the cortical layer in activation. The results already described indicate that calcium ions are not necessary in the primary phase of the activation process. Some change happens in the Ca-free condition in response to stimulating agent, but the breakdown of the cortical alveoli does not occur or occurs with difficulty in Ca-free media. This is clearly shown in the stimulation of oxalated eggs. The presence of calcium ions is necessary or at least has a favorable influence in the secondary phase of activation, in which the wave-like breakdown of the cortical alveoli takes place.

Heilbrunn's general theory (1) of stimulation in cells requires Ca release from the cortical protoplasm in the primary phase of stimulation. In order to determine whether or not Ca release occurs in our case, the following experiments have been done. When ripe unfertilized eggs are pricked with a fine glass needle whose tip is 15 μ in diameter, they are activated but no exudate is formed at the point of pricking. When the unfertilized eggs are pricked with a needle, the tip of which is 30–40 μ in diameter, yolk is always extruded from the region of pricking. If pricking is done on the cortical layer with a glass needle, the tip of which is 80 μ in diameter, an exudation of the cortical protoplasm can be secured. This is not always successful, since yolk is extruded if the needle is inserted into the yolk through the cortical protoplasmic layer.

Pricking with a needle causes stimulation and results in the wave-like breakdown of the cortical alveoli, followed by the separation of the egg membrane. This occurs also in Ca-free Ringer's solution, unless the eggs are washed with the solution several times during a period of more than 30 minutes. As is well known (13), the yellowish brown solution of sodium alizarin sulfonate forms purplish-red calcium alizarinate with free calcium ion. Pollack (13) used the color test to demonstrate the presence of free calcium ions in the living *Amoeba*. This color test has been used in our experiments.

Ripe unfertilized eggs kept in Ringer's solution were washed once with Ca-free Ringer's solution. They were immersed in a 0.3 per cent solution of sodium alizarin sulfonate (Grübler's) in M/7.5 Ca-free Ringer's and then pricked in the cortical layer with a glass needle, the tip of which was 80 μ in diameter. Care was taken not to prick into the yolk. In successful experiments, an exudate of cortical protoplasm is formed, which instantly becomes deep red, indicating the formation of Ca-alizarin sulfonate (Fig. 1, A). At the same time the wave-like breakdown of the cortical alveoli takes place. It has been shown (19) that when the eggs are anesthetized with M/200 chloretone-Ringer or M/200 phenyl urethane-Ringer for an hour, they

reversibly lose the capacity for response to pricking. When the same experiments are performed on eggs anesthetized with chloretone or phenyl urethane, the exudate of cortical protoplasm becomes only slight red (Fig. 1, B). The formation of Ca-alizarin sulfonate is very slight in anesthetized eggs. In this case no cortical change occurs, as is shown in Fig. 1, B. In these experiments care must be taken so that no yolk is contained in the exudate, since exudate containing yolk instantly becomes purplish-red in both normal and anesthetized eggs.

These experiments obviously indicate that when unfertilized eggs are stimulated by pricking, free calcium ions are released from the cortical protoplasm of the egg.

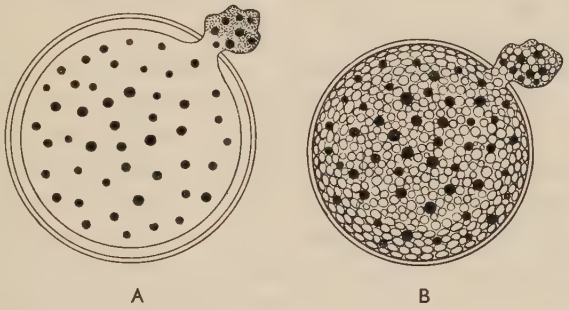

Fig. 1 A: An exudate of cortical protoplasm of an unfertilized *Oryzias* egg is produced in a solution of sodium alizarin sulfonate-Ringer. The exudate instantly becomes deep red, indicating the formation of calcium alizarin sulfonate (dots), and the cortical alveoli are broken down in a wave-like manner from the site of pricking. B: The same experiment with an egg anesthetized with chloretone-Ringer. The formation of calcium alizarin sulfonate (dots) is very slight and the cortical alveoli remain unchanged. The cortical alveoli are represented as white circles and the oil droplets as black circles.

DISCUSSION

The ripe unfertilized eggs of the medaka (*Oryzias latipes*) furnish a unique material for the study of fertilization and activation processes in teleostean eggs. The majority of the unfertilized eggs remain in a normal condition and retain the capacity for fertilization and activation for a long period in isotonic Ringer's solution. Only a few eggs (5–6 per cent) undergo autoactivation in the Ringer's solution. It has been shown that autoactivation is inhibited in Ca-free media. In an isotonic oxalate solution or in oxalated Ringer's solution, no eggs undergo autoactivation.

It has been known that the ripe unfertilized eggs of many freshwater fish undergo separation of the egg membrane when they are immersed in fresh

Ca release from the cortical layer in activation. The results already described indicate that calcium ions are not necessary in the primary phase of the activation process. Some change happens in the Ca-free condition in response to stimulating agent, but the breakdown of the cortical alveoli does not occur or occurs with difficulty in Ca-free media. This is clearly shown in the stimulation of oxalated eggs. The presence of calcium ions is necessary or at least has a favorable influence in the secondary phase of activation, in which the wave-like breakdown of the cortical alveoli takes place.

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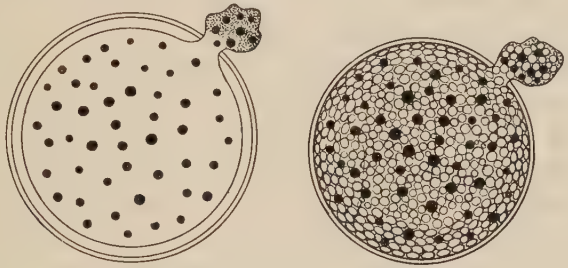

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DISCUSSION

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It has been known that the ripe unfertilized eggs of many freshwater fish undergo separation of the egg membrane when they are immersed in fresh

water. This may be regarded as activation, since such eggs show the cortical changes before membrane separation. This is the case with the eggs of the goldfish (*Carassius auratus*) and the pond smelt (*Hypomesus olidus*). It was found (unpublished experiments of the writer) that these eggs undergo cortical changes followed by membrane separation even in M/7.5 Ringer's solution. If the unfertilized eggs of these fish are immersed in M/11 sodium oxalate solution, no eggs undergo the cortical changes. When oxalated eggs are transferred to Ringer's solution or to fresh water within thirty minutes, they undergo typical activation. This shows that sodium oxalate reversibly inhibits the cortical changes in these eggs. In the case of *Oryzias latipes*, only a few eggs undergo auto-activation in Ringer's solution. This autoactivation is inhibited by sodium oxalate. It is obvious that calcium ions are necessary in auto-activation in teleostean fishes.

As has been mentioned above, when unfertilized *Oryzias* eggs are treated with an isotonic solution of sodium oxalate, they show no breakdown of the cortical alveoli on stimulation by pricking. Since these eggs undergo cortical changes on return to Ringer's solution without further stimulation, we are forced to consider that some event occurs in oxalated eggs when they are stimulated by pricking. In this respect oxalated eggs differ fundamentally from anesthetized eggs, which also fail to respond to stimulation, but no cortical change occurs in Ringer's solution. It requires further stimulation in order to induce the cortical change. It may be postulated that activation involves a series of processes which are connected in a catenary way. To account for the stimulation in the oxalated eggs it may be postulated that calcium ions are not necessary in the primary phase but that they are necessary in the secondary phase of the cortical response. Recent physiological studies favor the view that stimulation and excitation can be separated in many irritable systems. Some invisible change may take place in oxalated eggs in response to pricking, but the wave-like breakdown of the cortical alveoli is inhibited in the Ca-free condition.

This idea may also account for the results on chemical stimulation in Ca-free media. Although some percentage of eggs shows the cortical response in the absence of calcium ions, the percentage of activated egg is very low as compared with eggs in Ringer's solution. The positive response of some eggs in Ca-free media may be attributed to the calcium ions released from the protoplasm on stimulation.

The reaction of the exudate of cortical protoplasm with sodium alizarin sulfonate indicates that free calcium ions are released from the cortical protoplasm in the activation process. In anesthetized eggs the formation of

calcium alizarinate is only slight. Heilbrunn *et al.* (2, 3, 4) using various irritable systems such as *Amoeba*, sea urchin eggs, etc. as materials, showed that the first process of stimulation is a release of free calcium ions from the cortex of the cells, and the second process is clotting of the interior protoplasm in a reaction with calcium ions. It is believed that ciliary reversal in *Paramecium* is a sign of excitation. Kamada (5, 6) has shown that this ciliary reversal is induced by the reduction of intracellular calcium ions. He was able to demonstrate the release of free calcium ions from the site of pricking with a micropipette by means of sodium alizarin sulfonate. He assumed the existence of a calcium compound and suggested that the release of calcium ions from this compound is the cause of excitation in *Paramecium*. Kamada (7) has also shown in the unfertilized eggs of a sea urchin that membrane elevation occurs when the concentration of intracellular Ca ions is suddenly reduced by intracellular injection of oxalate or citrate. Mazia and Clark (10) performed a clear-cut experiment on plant cells (*Elodea*), which induce Ca release from the protoplasm.

As has been shown in previous papers, the most prominent feature in fertilization and activation of *Oryzias* egg is a wave-like breakdown of cortical alveoli which are embedded in the cortical protoplasm in the unfertilized egg (14, 16). In fertilization, the breakdown of the cortical alveoli begins near the animal pole and ends at the vegetal pole. This change is subsequently followed by the separation of the egg membrane. Experiments indicate that a water-soluble colloid is released from the cortical alveoli and that the egg membrane is separated from the surface by the colloid osmotic pressure of the alveolar colloid, to which the egg membrane is impermeable. Recently the writer was able to observe release of the colloid from the cortical alveoli at the time of their breakdown.

Essentially similar cortical changes are provoked by means of chemical (surface active substances) and thermal stimulation. In electric (galvanic) stimulation, the cortical changes begin at the anodal and cathodal sides and pass along the egg surface (18). When unfertilized eggs are pricked at the equator, the change begins from the point of stimulation and propagates along the surface of the eggs, but does not end at the antipode. The wave of breakdown of the cortical alveoli propagates more rapidly in the animal than in the vegetal region, so that it ends at a point half way between the antipode and the vegetal pole. Pricking and electrical stimulation indicate that there is a gradient of excitation-conduction which is the highest at the animal pole and the lowest at the vegetal pole (17, 18).

As to the mechanism of the wave-like breakdown of the cortical alveoli,

two possibilities can be considered. One is that breakdown of one cortical alveolus itself causes the breakdown of the neighboring cortical alveoli. An alternative is that an invisible wave of some sort, which causes the visible breakdown of the cortical alveoli, propagates along the cortical protoplasm, regardless of the presence or absence of cortical alveoli. That the latter alternative is correct was proved by centrifuging experiments. In centrifuged eggs, the cortical alveoli are shifted towards the centrifugal part of the eggs. In this case they are present in both the centrifugal part of the cortical protoplasm and of the yolk. The eggs in which the animal hemisphere is alveoli-free can be fertilized. In this case the cortical alveoli which are contained in the cortical protoplasm are broken down, and the separation of the egg membrane follows. The alveoli which were forced to move into the yolk remained unchanged. The same is true when the alveoli-free cortical protoplasm of centrifuged eggs is pricked with a fine needle. These experiments indicate that an invisible wave of some sort, which causes the breakdown of the cortical alveoli, propagates along the cortical protoplasm. It cannot pass through the yolk. This invisible wave which causes the visible breakdown of the cortical alveoli has been designated by the writer as the "fertilization-wave" (17). It is this invisible wave which propagates along the cortical protoplasm as a true physiological wave. The visible wave of the breakdown of the cortical alveoli is the result of the fertilization-wave. It has been postulated, on the basis of the anesthesia experiments that the fertilization-wave may be regarded as a wave of excitation. The experiments described above indicate that pricking with a fine needle fail to set up a fertilization-wave in oxalated eggs. Since these eggs reveal the wave-like breakdown of the cortical alveoli on return to Ringer's solution without further stimulation, some sort of change must have occurred in oxalated eggs on pricking in oxalate solution. Somewhat similar result are seen following chemical stimulation in oxalated eggs.

It may be postulated that the first phase (stimulation) of activation occurs in oxalated eggs, but the second phase (excitation) or the fertilization-wave is inhibited. The cortical changes in *Oryzias* eggs induced by activating agents may be expressed in the following catenary reaction:

> Stimulants → stimulation → fertilization-wave → breakdown of the cortical alveoli → release of colloid from alveoli → membrane separation.

In the stimulation process no calcium is necessary. In this phase free calcium ions are released from the cortical protoplasm, as is shown by the color test with sodium alizarin sulfonate. Calcium ions may be necessary for the

establishment of the fertilization-wave. It has been shown (14, 16) that fertilization is inhibited in Ca-free media or in oxalate solution. Since the spermatozoon acts as the stimulant in fertilization, the same processes may be involved in the fertilization process. In fertilization, however, union of the spermatozoon with the cortical protoplasm of egg is necessary before the establishment of stimulation. Calcium ions may also be necessary for the union of the gametes.

SUMMARY

1. A few unfertilized eggs (5–6 per cent) of the medaka (*Oryzias latipes*) reveal autoactivation in the isotonic Ringer's solution. The percentage of autoactivation markedly decreases in Ca-free Ringer's solution. No eggs show autoactivation in an isotonic oxalate solution or in oxalated Ringer's solution.

2. When unfertilized eggs are treated with isotonic oxalate solution they lose the capacity for cortical changes on stimulation by pricking. These eggs show cortical response without further stimulation when transferred to Ringer's solution.

3. The presence of calcium ions favors the cortical response of eggs on stimulation with sodium oleate and saponin. Only a few eggs show activation if the eggs are first treated with these chemical agents dissolved in Ca-free Ringer or in oxalate solution and then transferred to Ca-free media.

4. The reaction of an exudate of cortical protoplasm with sodium alizarin sulfonate indicates that free calcium ions are released from the cortical protoplasm at the time of stimulation with pricking. Anesthetized eggs reveal only a slight reaction with the dye.

5. Calcium ions are not necessary in the primary phase of activation; they are released from the cortical protoplasm at this phase. Calcium ions are necessary or at least have a favorable effect in the secondary phase of activation.

6. The cortical changes involved in activation of *Oryzias* eggs may be expressed by the following catenary reactions: Stimulants → stimulation → fertilization-wave → breakdown of cortical alveoli → release of colloid from alveoli → separation of egg membrane. Calcium ions are released in stimulation and they may be necessary for the establishment of the fertilization-wave which causes the breakdown of the cortical alveoli.

I am grateful to Dr. J. Clark Dan for her kind help in improving the manuscript.

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Experimental Cell Research, **6**, *69–75 (1954)*

INCORPORATION *IN VITRO* DE GLYCINE-1-^{14}C DANS LES CELLULES INDIVIDUELLES DE LA MOELLE OSSEUSE

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Reçu le 27 Avril, 1953

La technique autoradiographique basée sur l'observation des traces individuelles émises par un élément radioactif (2) offre sur la méthode classique, où l'activité se mesure par le noircissement, plusieurs avantages appréciables: elle permet, en effet, de localiser avec précision l'élément en question, pour autant que l'émulsion sensible soit en contact intime avec la préparation histologique, de mesurer la valeur relative des phénomènes par le comptage des particules émises, et enfin d'éviter certaines confusions dues à des artéfacts: la précipitation parasite des grains d'Ag par des substances réductrices pouvant exister dans les tissus ou la pigmentation naturelle de certaines préparations, par exemple.

Il nous a paru utile de mettre au point une technique basée sur ce principe et de l'appliquer à un matériel biologique bien connu et relativement facile à utiliser: c'est le cas de la moelle osseuse (5).

Altmann *et al.* ont démontré l'existence d'une incorporation de glycocolle, marqué au ^{14}C, dans les cellules de la moelle osseuse (1). Boyd *et al.* (3) ont donné la preuve autoradiographique de ce phénomène, en étudiant le noircissement de l'émulsion par les cellules individuelles. La même technique a été appliquée par Morse (8) en utilisant cette fois du ^{32}P.

MATÉRIEL ET MÉTHODES

Dans la présente expérience, des fragments de moelle osseuse sont prélevés dans les os longs d'un rat. Les cellules sont dispersées dans du Ringer citraté dépourvu de Ca^{++}, à l'aide d'un homogénéiseur en verre du type Potter. La suspension cellulaire est filtrée sur de la gaze, centrifugée pendant 5 minutes à 2.500 t/m et remise en suspension dans la même solution. L'incubation a lieu à 37°, en présence de glucose isotonique et de tampon au phosphate $\frac{M}{30}$ à pH 7.5. La suspension contient environ, par mm^3, 10^6 cellules et 150 γ de glycocolle, dont 60 % des atomes de carbone du carboxyle sont marqués.

Après incubation, la suspension est diluée de manière à obtenir une dispersion cellulaire satisfaisante sur des lames préalablement gélatinées; les frottis sont ensuite fixés à l'alcool méthylique. Ils sont recouverts d'émulsion Ilford G 5 «in gel form» diluée au tiers par de l'eau bidistillée, fondue à 45° puis refroidie aux environs de 35°: à cette température, le dommage causé aux cellules est minime et l'on peut éviter l'emploi d'un film protecteur (formvar, plexiglas) qui diminue la résolution. L'épaisseur de l'émulsion sèche est estimée à 40 μ environ après fixation.

Nous avons procédé au développement photographique après des expositions de 6 et de 12 heures; l'incubation étant effectuée dans un milieu fortement radioactif, une durée brève d'exposition est suffisante pour que l'observation soit aisée et que les électrons du back-ground puissent être négligés.

Après le développement au révélateur Kodak X, fixation dans une solution d'hyposulfite de Na $^1/_3$ saturée et lavage soigneux à l'eau courante, les préparations sont colorées suivant la technique habituelle de May Grünwald-Giemsa. Dans les cas où le temps de coloration n'a pas été trop prolongé (4 minutes dans le bleu de méthylène-éosine $^1/_3$, suivi de 15 minutes dans l'azuréosine dilué dans l'eau au $^1/_{20}$e), l'identification des cellules est parfaitement possible malgré la couche de gélatine et la longue incubation *in vitro*.

RÉSULTATS EXPÉRIMENTAUX

L'expérience décrite dans le présent travail a porté sur l'examen d'environ 800 cellules de chaque lame après respectivement 6 et 12 heures d'exposition (le temps étant compté à partir du moment où l'émulsion est sèche).

Les résultats sont rassemblés dans le tableau I et dans le graphique I. Quelques microphotographies permettent de juger de l'état des cellules (Figs. 1, 2, 3).

On constate, tout d'abord, que le pourcentage des cellules marquées est deux fois plus considérable dans le frottis exposé pendant 12 heures que dans celui exposé pendant 6 heures seulement.

En ce qui concerne la série granulocytaire, on assiste à une diminution considérable de la radioactivité des cellules au cours de leur maturation. Pour donner une idée de la radioactivité spécifique de chaque cellule, leur diamètre a été mesuré: il varie de 10,2 μ pour les cellules les plus actives à 7,3 μ pour les moins actives, ce qui correspond à une variation de volume de l'ordre de 3 à 1 (cfr tableau I). Si on ramène les activités par cellule à une valeur corrigée pour le volume individuel de chaque cellule, on obtient une évaluation approximative de «l'activité spécifique». On suppose, dans ce cas, que les cellules conservent sur le frottis une forme sphérique, ce qui correspond à la correction maxima. La valeur ainsi obtenue n'est évidemment qu'une valeur approchée, puisqu'il n'est pas tenu compte de la densité des cellules. Une méthode basée sur une mesure interférométrique

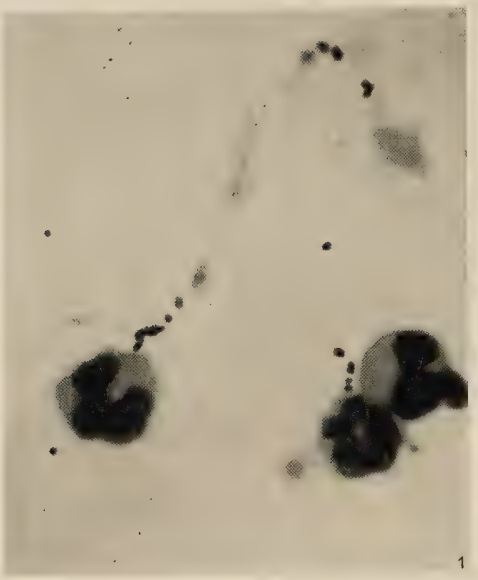
TABLEAU I

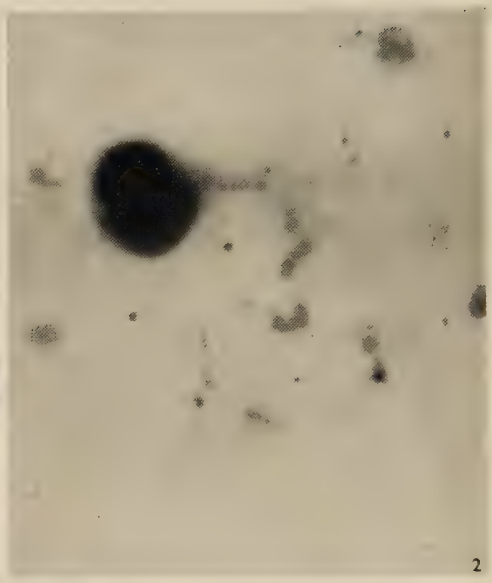
	Volume μ^3	Exposition 6 h.				Exposition 12 h.			
		Nb cell.	Nb traces	Trace / cell. $\times 10^2$	«Activ. spéc.»[1]	Nb cell.	Nb traces	Traces % cell.	«Activ. spécif.»[1]
Myéloblastes	557	2				3	6		
						(6)	(12)	200	360
Promyélocytes	510	12	6	50	98	4	7		
						(21)[2]	(22)	116	228
Myélocytes									
neutrophiles	425	86	52	67	158	50	63	126	295
éosinophiles		2	1						
Métamyélocytes									
neutrophiles	236	185	66	36	152	119	87	73	310
éosinophiles	353	49	18	37	103	24	17	70	195
Leucocytes									
neutrophiles	209	212	24	125	60	164	49	30	144
éosinophiles	310	60	12	20	65	55	30	54	175
Erythroblastes									
basophiles						1			
polychrom.		3				8	5		
						(8)	(4)	56	
orthochrom.		8	3	37		18	11		
						(21)	(8)	49	
Hématies +									
réticulocytes		180	4	2.2		135	5	3.7	
Prolymphocytes		3	1			8	9	115	
Lymphocytes		88	11	12.5		170	44	26	
Histiocytes jeunes						2	4		
Plasmocytes						2	1		
Megacaryocytes						1	2		
Total[3]		891	198	22[2]		785	362	46	

[1] «Activité spécifique»: $\frac{\text{traces par cellule}}{\text{vol. cellule}} \times 100$.

[2] Les chiffres entre parenthèses proviennent de cellules comptées isolément. Les autres ont été déterminées au hasard comme on le fait dans le cas des myélogrammes habituels.

[3] Dans le total ne sont pas comptés les chiffres entre parenthèses.





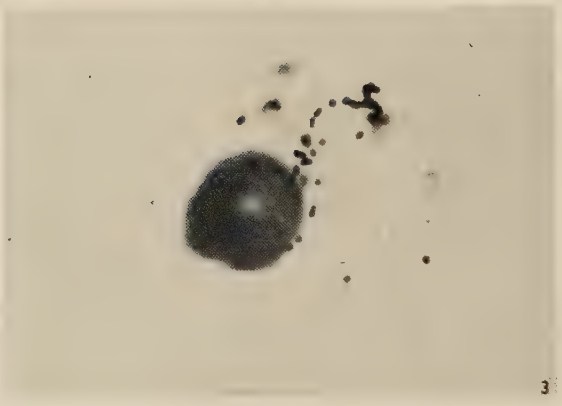


Fig. 1. Trois leucocytes polynucléaires; le plus petit est un neutrophile, les deux autres sont des éosinophiles.
Fig. 2. Un normoblaste orthochromatique.
Fig. 3. Un myéloblaste.

des constituants cellulaires (4) serait certainement plus exacte. Notons que cette correction due aux variations de volume cellulaire modifie assez fortement l'allure générale du phénomène observé, comme on peut s'en rendre compte en comparant les graphiques I A et II. Ajoutons que l'on n'a pas pris en considération l'absorption des électrons au sein des cellules elles-mêmes. Cette absorption est d'autant plus considérable que le diamètre cellulaire est plus grand: une telle correction, dans le cas qui nous préoccupe, aurait plutôt tendance à augmenter la radioactivité spécifique des cellules les plus volumineuses.

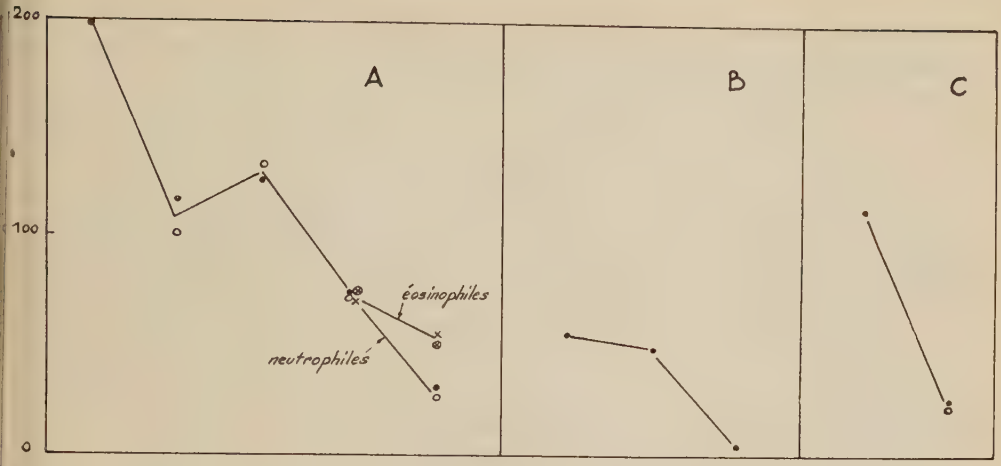


Fig. I.

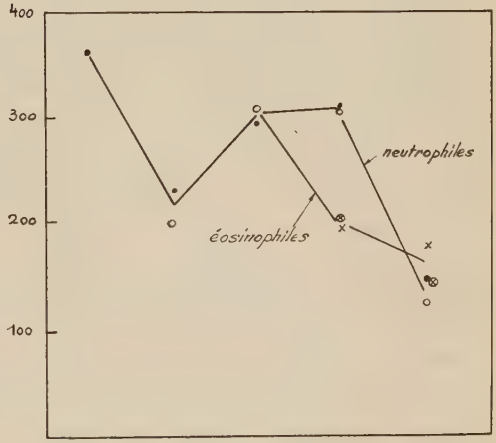


Fig. II.

Fig. I. Radioactivité des divers types de cellules de la moelle osseuse au cours de leur maturation. Ordonnées: nombre de traces par cellule $\times 10^2$: $\times$ ●, après 12 heures d'exposition; ⊗ ○, après 6 heures d'exposition (résultats $\times 2$).
Fig. II. « Activité spécifique » de la lignée leucocytaire. Ordonnées: nombre de traces par cellule $\times 10^2$: $\times$ ●, après 12 heures d'exposition; ⊗ ●, après 6 heures d'exposition (résultats $\times 2$).

Les phénomènes observés sont fort semblables dans le cas des cellules de la série lymphocytaire et dans celui des globules rouges. Dans la lignée rouge, les érythroblastes présentent une incorporation encore assez importante, tandis que les érythrocytes arrivés à maturité n'ont qu'une activité négligeable qui peut probablement être attribuée aux réticulocytes.

Ces constatations concordent bien avec les résultats des travaux de Altman (1), Shemin, London et Rittenberg (7, 9) et Chantrenne et Koritz (6) établissant que la synthèse de l'hémoglobine n'est plus possible dans les

érythrocytes non nuclés alors qu'elle l'est encore dans les réticulocytes et les cellules plus jeunes. Cette synthèse débute au stade proérythroblaste et a lieu au cours de toute la maturation (10).

DISCUSSION

Nos résultats sont, dans une certaine mesure, comparables à ceux de Morse, obtenus au moyen de ^{32}P; toutefois, dans le cas présent, on observe une activité non négligeable des leucocytes mûrs. Dans le travail de Morse, il n'y a d'activité leucocytaire que si l'animal a été injecté 21 heures avant la préparation des frottis. Mais la technique de noircissement utilisée par cet auteur, qui n'était pas suivie d'un comptage des grains ou d'une étude photométrique, est très loin d'être quantitative.

Les constituants marqués dans les cellules au cours de l'incorporation de la glycine-1-^{14}C sont vraisemblablement des protéines ou des groupesp rosthétiques qui leur sont associés (acides nucléiques, cytochromes, hème). Un fractionnement cytochimique devrait permettre de préciser ce point (5). D'autre part, l'utilisation de précurseurs marqués, plus spécifiques de certains groupements, devrait fournir des renseignements plus précis encore. Des expériences analogues à l'aide d'acide orotique marqué (précurseur des pyrimidines) sont en cours et elles donneront vraisemblablement d'utiles renseignements sur la synthèse des acides nucléiques. De même, l'utilisation d'inhibiteurs compétitifs marqués pourrait peut-être permettre également une localisation cytochimique d'un enzyme donné à condition que ces inhibiteurs soient suffisamment spécifiques.

Nous n'avons pas tenté, dans le présent travail, de localiser le point d'origine de chaque trace. De telles observations sont certainement possibles à l'aide de la technique utilisée. Notons toutefois que les érythroblastes ont montré un certain nombre de traces issues du noyau, d'autres du cytoplasme. Dans le cas des myéloblastes, des traces ayant leur origine dans le nucléole ont été observées avec certitude, dans un nombre non négligeable de cas. Cependant, il serait certainement préférable d'opérer sur des coupes suffisamment minces au lieu de frottis, afin d'éviter toute incertitude du fait des constituants cellulaires situés au dessus ou en dessous de l'organite qui paraît émettre l'électron. Le choix de cellules de taille plus grande que celles de la moelle osseuse facilitera beaucoup ce travail de localisation. Des expériences sur des amibes et des cellules embryonnaires sont d'ailleurs actuellement effectuées par l'un de nous (A. F.).

Nous pensons que la technique que nous avons mise au point se révélera

d'une très grande utilité car elle permettra de mesurer avec une assez bonne précision l'activité spécifique des divers types de cellules; elle complétera ainsi utilement toute étude du métabolisme d'un tissu complexe au moyen d'isotopes. Précisons enfin, que la méthode utilisée est susceptible en principe de donner des résultats en valeur absolue: connaissant le nombre de traces, la durée d'exposition et la période de l'isotope, il est possible de calculer, par cellule, le nombre de molécules incorporées; mais, comme nous l'avons fait remarquer, les corrections dont il faut tenir compte devront faire l'objet d'une étude minutieuse.

RÉSUMÉ

Une technique autoradiographique basée sur l'observation des traces individuelles émises par un élément radioactif au sein d'une cellule a été mise au point en ce qui concerne les cellules de la moelle osseuse. Les résultats obtenus au moyen de glycine-1-C_{14} ont montré une incorporation particulièrement intense au sein des cellules jeunes; l'incorporation diminue progressivement au cours de la maturation, pour devenir presque nulle dans le cas des hématies. Les leucocytes polynucléaires et les lymphocytes mûrs sont le siège d'une incorporation non négligeable.

Toute notre gratitude va au Professeur G. Occhialini qui nous a guidés dans l'application de la technique autoradiographique.

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ÉTUDE AU MICROSCOPE ÉLECTRONIQUE DES TRACHÉES DES LARVES D'INSECTES[1,2]

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Reçu le 29 Avril, 1953

Les résultats présentés dans ce mémoire font partie d'une étude générale de la structure submicroscopique des glandes séricigènes des larves de *Galleria mellonella*. Nous nous bornerons ici à la description du squelette chitineux du système trachéen qui doit assurer l'aération des glandes séricigènes.

La chitine a été étudiée au microscope électronique, dans le cas de la cuticule de *Periplaneta*, par Richards et Anderson (1).

MATÉRIEL ET MÉTHODES

Nous avons choisi les glandes séricigènes provenant de larves de *Galleria mellonella* qui s'apprêtaient à filer leur cocon, c. à. d. de larves ayant atteint la grandeur maxima. Les glandes, avec les trachées adhérentes, ont été enlevées, lavées et ensuite séchées de façon à ce que non seulement les structures rigides (chitine), mais aussi les structures plus délicates (forme générale de la glande) ne s'altèrent que dans des limites restreintes. Ce procédé de séchage est indispensable pour la prise des empreintes ou «répliques» au méthylmétacrylate.

Le microscope électronique utilisé dans nos recherches était du type Philips EM 100 et la tension fut maintenue à 100 kV.

Comme la question des artéfacts est de la plus haute importance en microscopie électronique, nous avons adopté le procédé de contrôle suivant: les glandes et les trachées furent observées par microprojection (grossissement $\times 500$) pendant toute la période de séchage. Nous avons photographié la glande immédiatement avant et après la prise de la réplique. Ensuite ces photos ont été comparées avec l'image obtenue directement de la réplique même. Ce procédé nous a permis de mettre en évidence les différences existant entre la structure extérieure et la structure interne de l'objet. En vue de l'examen au microscope électronique, ces répliques ont été recouvertes d'une fine couche de monoxyde de silicium. Cette couche fut ensuite détachée et ombrée au palladium.

DISCUSSION DE QUELQUES RÉSULTATS PROVENANT DE L'EXAMEN DES MICROGRAPHIES ÉLECTRONIQUES

Il résulte d'un examen général des photos que le système trachéen au niveau des glandes séricigènes se compose d'un réseau de trachées dont le

[1] Ce travail fut présenté sous la forme d'une brève communication à la seance de travail du 17 février 1953, de la Soc. belge de Biochimie (réunion de Gand).

[2] Travail exécuté avec le microscope électronique de l'Irsia.

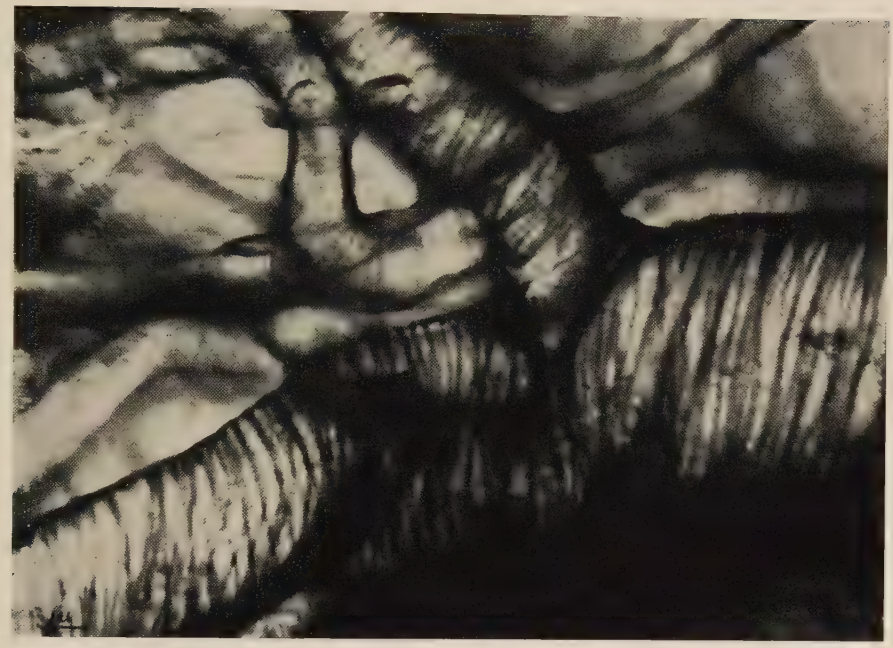

Fig. 1. Trachées et trachéoles se trouvant sur la paroi extérieure de la glande séricigène de *Galleria mellonella*. Methylmétacrylate-SiO; ombrage au Pd; grossissement ×6000.

diamètre diminue à chaque ramification pour former les trachéoles, qui s'engagent finalement dans la paroi extérieure (tunica propria) de la glande filifère (fig. 1). Un phénomène frappant est celui de la configuration des spirales ou anneaux chitineux qui assurent la rigidité des trachées et trachéoles : ces spiralisations sont multiples dans le cas des trachées de diamètre supérieur à 2 μ, tandis que celles des trachéoles dont le diamètre varie entre 1,5 et 0,5 μ se distinguent par un motif simple, régulier et facile à suivre. Nous avons réussi à démontrer que ces spiralisations traversent en réalité la paroi des trachées et des trachéoles (fig. 2).

Un fort grossissement a permis d'observer en plus des spiralisations, un réseau très fin unissant les spirales (fig. 3), et qui semble indiquer une structure polyédrique comportant un grand nombre de « nœuds » dont la signification reste encore obscure.

La présence de spiralisations dans les trachéoles qui se montrent moins compliquées que dans les trachées, a été déniée par certains auteurs (2). Il ressort pourtant de nos photographies que l'existence d'une spiralisation chitineuse dans les trachéoles est incontestable. Elle reste visible même aux endroits où la trachéole s'introduit dans le tissu glandulaire.

Fig. 2. Trachéoles coupées montrant l'intérieur de leurs parois. Méthylmétacrylate-SiO; ombrage au Pd; grossissement ×10000.

RÉSUMÉ

1. L'étude au microscope électronique révèle des différences dans la structure des spirales chitineuses du système trachéen: les trachées dont le diamètre est supérieur à 2 μ sont pourvues d'une spiralisation multiple, d'un aspect plutôt compliqué, tandis que les trachéoles dont le diamètre varie entre 0,5 et 1,5 μ sont caractérisées par la présence d'une spiralisation simple.

2. Cette spiralisation simple et uniforme se maintient tout le long de la trachéole.

3. Au point de vue technique, il convient de souligner les avantages qu'offre la méthode des répliques pour l'étude de structures chitineuses au microscope électronique.

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Experimental Cell Research, **6**, 79–86 (1954)

EINE EINFACHE DURCHSTRÖMUNGSAPPARATUR ZUR GEWEBEZÜCHTUNG AUS NICHTROSTENDEM STAHL

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Eingegangen am 17. Mai 1953

Die im nachstehenden beschriebene Apparatur wurde entwickelt, um Gewebezellen ausserhalb des Körpers unter möglichst konstanten bzw. kontrollierbaren Bedingungen über lange Zeiträume bei bestmöglichen optischen Gegebenheiten mikroskopieren zu können. Es sollte im besonderen der Einfluss eines konstanten oder experimentell veränderten Mediums auf die Zellen beobachtet werden. Gleichzeitig sollte mit Hilfe des Zeitrafferfilms das Verhalten der Zellen verfolgbar sein.

Dazu eignete sich von vornherein keine der üblichen Methoden Gewebe im Deckglaspräparat, in der Carrel-Flasche oder ähnlich zu züchten, weil entweder keine stärkere mikroskopische Vergrösserung anwendbar ist, oder der notwendige Wechsel des Nährmediums eine Unterbrechung der Beobachtung bedeutet.

Alle derartigen Versuche werden dagegen leicht zu bewältigen sein, wenn man das zu untersuchende Gewebe in einer gut mikroskopierbaren Beobachtungskammer hält, die von einem flüssigen Nähr- oder Untersuchungsmedium langsam durchströmt wird. Die Flüssigkeit, die aus einem grösseren Reservegefäss kommt, kann entweder abfliessen, wenn sie die Beobachtungskammer passiert hat, oder sie kann mit Hilfe eines Pumpmechanismus wieder in das Reservegefäss zurückbefördert werden. Indem man sie hier wieder etwas regeneriert, z. B. durch Wiederherstellen einer gewünschten, konstant zu haltenden Gasspannung, lässt sich so ein Kreislauf der Nährflüssigkeit erzielen. Abgesehen davon, dass im Gegensatz zu allen anderen Methoden nur in einer solchen Durchströmungskultur den Geweben ein annähernd konstantes Milieu geboten werden kann, bietet sich auch die Möglichkeit die Durchströmungsflüssigkeit im Reservegefäss einer dauernden Kontrolle in physikalisch-chemischer Hinsicht zu unterziehen bzw. experimentelle Änderungen genau zu dosieren und zu verfolgen.

Wohl aus ähnlichen Erwägungen im Hinblick auf diese Vorteile hat man sich schon recht früh mit der Konstruktion solcher Durchströmungsapparate

befasst. Es sei hier nur auf die entsprechenden Arbeiten von Burrows, Romeis, Carrel, Lindbergh, de Haan, Schade und anderen hingewiesen (1–7).

Alle diese Einrichtungen haben jedoch keine verbreitete Anwendung gefunden. Sie bestanden aus Glas, und die einzelnen Teile mussten oft getrennt sterilisiert und dann mit Gummischlauch zusammengesetzt werden. Hierdurch wird die Infektionsgefahr relativ gross, was besonders ins Gewicht fällt, weil man wegen des erforderlichen Zeit- und Materialaufwandes meist nur wenige Apparaturen in Betrieb nehmen kann. Zudem war eine einwandfreie mikroskopische Beobachtung der Kulturen während der Durchströmung wegen der hierfür ungünstigen Bauart der Kammern nicht möglich.

Es lag deshalb nahe, das in konstruktiver Hinsicht wenig geeignete Glas durch ein anderes Material zu ersetzen, das bei günstigeren mechanischen Eigenschaften gleichzeitig physiologisch ebenso indifferent wie dieses ist. Wenn sich auch noch nichts abschliessendes über die physiologische Unschädlichkeit des verwendeten nichtrostenden Stahles (Remanit 1880 SST der Deutschen Edelstahlwerke) sagen lässt, und er in manchen Fällen eine schädliche Wirkung entfalten sollte, so bleibt immer die Möglichkeit ein ähnliches Material an seiner Stelle bei gleicher Konstruktion zu verwenden. Es wird dabei an Edelmetalle bzw. deren Legierungen gedacht, die in vieler Hinsicht erprobter, dafür aber kostspieliger sind. Die Verwendung von Metall erlaubt eine sehr einfache Ausführung der Beobachtungskammer, wie sie in Glas so leicht und billig nicht herstellbar ist. Ferner können die Verbindungen zwischen Vorratsgefäss und Beobachtungskammer mit Hilfe von Metallröhrchen starr sein, so dass keine Gummi als Verbindungsmaterial notwendig wird. Die Apparatur wird *in toto* sterilisierbar, was wesentlich einfacher und mit geringerer Infektionsgefahr verbunden ist, als wenn verschiedene Teile einzeln sterilisiert und dann zusammengesetzt werden müssen. Da die erforderlichen Stahlröhrchen käuflich sind, lässt sich die ganze Einrichtung von jedem Institutsmechaniker herstellen.

Im einzelnen besteht die Apparatur (Siehe Abb. 1) aus einem Vorratsgefäss (VG), einem darunter befindlichen runden Stahlteil (P), auf welchen das Vorratsgefäss aufgeschraubt ist, den beiden Rohrleitungen zur Kammer und schliesslich der Kammer (K) selbst. Alle Teile sind zusammen auf eine einfache Halterung aus Aluminium montiert, an der gleichzeitig ein Luftfilter (L) angebracht ist. Mit Hilfe eines Kugelgelenkes (KG) ist die Apparatur an einem Stativ befestigt.

Das Vorratsgefäss hat die Form eines Becherglasses, dessen Boden in der Mitte ein Loch von 15 mm Durchmesser hat. Bei einer Höhe von 80 mm und

einem Durchmesser von 45 mm ist es zur Aufnahme von etwa 100 cm^3 Flüssigkeit gedacht. Verschlossen wird es nach oben durch einen einfachen Überwurf aus Glas.

Dieses Vorratsgefäss sitzt auf dem bereits erwähnten zylinderischen Stahlstück, das oben einen kurzen, in die Bodenöffnung des Glases passenden Ansatz hat. Dieser Ansatz ist mit einem nicht zu groben Gewinde für eine

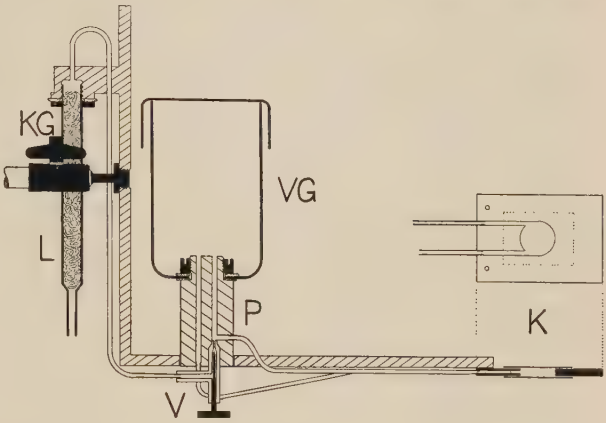

Abb. 1. Schematische Darstellung der Apparatur im Längsschnitt. VG: Vorratsgefäss, P: Pumpenteil, K: Kammer, V: Gasventil, KG: Kugelgelenk, L: Luftfilter.

flache Schraubenmutter versehen, mit deren Hilfe unter Verwendung dünner Asbestdichtungen das Vorratsgefäss auf dem Stahlteil anzuschrauben ist. Da die Schraubenmutter am Grunde des Vorratsgefässes schlecht mit der Hand erreicht werden kann, wird sie mit Hilfe eines Spezialschlüssels angezogen. Der hier verwendete trägt an einem Stiel eine Scheibe mit zwei, in entsprechende Bohrungen der Mutter passenden Zapfen.

Die mit ihrem unteren Teil in der Aluminium-Halterung festsitzende, das Vorratsgefäss tragende massive Stahlwalze von etwa 30 mm Höhe enthält zwei durchgehende Bohrungen, in denen die durch aufstiegende Gasblasen transportierte Flüssigkeit strömt. Man kann deshalb diesen Teil als Pumpenteil bezeichnen. Von den beiden Bohrungen des Pumpenteiles führt die eine aus dem Vorratsgefäss weiter in ein unten eingebrachtes Stahlrohr direkt zur Beobachtungskammer. Das zweite, aus der Kammer zurückführende Rohr mündet seitlich, etwa 12 mm über dem unteren Ende des Pumpenteils, in dessen zweite Bohrung ein. Diese Bohrung ist von unten bis zur seitlichen Einmündungsstelle der Rückleitung aus der Kammer durch einen Einsatz verschlossen. Wie aus Abb. 1 ersichtlich, stellt dieser Einsatz ein einfaches Nadelventil (V) dar, aus dessen feiner Öffnung das eingeleitete Gas bzw. Gasgemisch in Gestalt kleiner Bläschen austritt. Jede der in dem engen

Rohr aufsteigenden Gasblasen hebt die über ihr stehende und durch das seitliche Rohr nachströmende Flüssigkeit in das Vorratsgefäss hoch. Durch das zweite Rohr fällt die Flüssigkeit dann wieder herab, so dass ein mit den Gasblasen leicht pulsierender, langsamer Flüssigkeitsstrom durch die Beobachtungskammer entsteht.

Diese einfache Methode die Flüssigkeit in Umlauf zu versetzen hat den Nachteil, dass man ein Gas durch die Nährflüssigkeit hindurchleiten muss, was nicht immer erwünscht und günstig ist. In sehr vielen Fällen wird dies jedoch nicht stören, und man hat dann den Vorteil eines zuverlässigen und erschütterungsfreien Betriebes, was für eine Dauerbeobachtung mit Hilfe des Zeitrafferfilms von grösster Wichtigkeit ist.

Durch Drehen der nach unten herausragenden Rändelschraube kann die durchströmende Gasmenge und damit die Geschwindigkeit des Flüssigkeitsstromes genau eingestellt werden. Die Gaszuführung zum Ventil geschieht durch ein Stahlröhrchen, welches senkrecht nach oben bis über den höchsten Flüssigkeitsstand im Reservegefäss geführt ist. Es soll so verhindert werden, dass eventuell Flüssigkeit infolge einer Unachtsamkeit durch das Gasventil in den angebauten Gasfilter gelangt.

Die eigentliche Beobachtungskammer ist eine planparallel geschliffene, 3 mm starke Stahlplatte mit einem runden Durchbruch von 16 mm Durchmesser. Seitlich münden in diesen Durchbruch zwei Bohrungen ein, die ihrerseits die beiden Röhrchen für die zu- und abfliessende Nährflüssigkeit aufnehmen. Oben und unten wird der Durchbruch durch aufparaffinierte Deckgläser verschlossen, so dass eine runde Kammer von 3 mm Höhe und 16 mm Durchmesser entsteht. Weil die zu mikroskopierenden Gewebe an die Unterseite des oberen Deckglases gebracht werden, sind sie im Hellfeld auch mit stärksten Objektiven erreichbar. Ferner lässt sich das Phasenkontrastverfahren anwenden. Verwendet man die Leitzsche Einrichtung, die wegen der speziellen Bauart des Phasenkondensors besonders geeignet ist, so sind die entsprechenden Objektive bis zu 70-facher Eigenvergrösserung benutzbar.

Die am Vorratsgefäss aufsteigende senkrechte Aluminiumplatte trägt in ihrer Mitte ein Loch mit Gewinde. Das hierin eingeschraubte Kugelgelenk ist mit einer kurzen Stange an einem kleinen Stativ befestigt (Abb. 2). Dergestalt hängt die ganze Durchströmungsapparatur also nur an diesem einen Kugelgelenk. Sie ist hierdurch nach jeder Richtung frei beweglich und mit Hilfe der Klemmschraube des Kugelgelenks in jeder gewünschten Lage zu fixieren. Dies ist einerseits bei der Inbetriebnahme der Apparatur von Bedeutung und andererseits nötig, um die etwa 12 cm seitlich frei herausragende Beobach-

tungskammer wirklich vollkommen parallel zum Mikroskoptisch ausrichten zu können.

Die Röhrchen zwischen Vorratsgefäss und Beobachtungskammer sind an den Verbindungsstellen in die entsprechenden Einsätze mit einem schwachen Konus hineingedrückt. Sie sind nicht federhart sondern unter Schutzgas etwas weichgeglüht, damit sie sich leichter biegen lassen.

Die Inbetriebnahme der Apparatur geht folgendermassen vor sich: Nach ihrer gründlichen Reinigung (der Stahl verträgt nötigenfalls auch eine Behandlung mit Säuren oder Laugen) werden besonders die dünnen Rohrleitungen kräftig mit dest. Wasser ausgespült und die Apparatur im Thermostaten getrocknet. Man lässt dazu am besten das Kugelgelenk samt einer kurzen Stange fest an der Apparatur verschraubt, weil sie sich dann schnell an einem Stativ befestigen und so leichter bedienen lässt. Als erstes wird dann das Vorratsgefäss mit den erwähnten dünnen Asbestdichtungen aufgesetzt und mit Hilfe des Spezialschlüssels nicht zu kräftig angeschraubt. Wird die Haltemutter zu fest angezogen, so springt das Glas leicht bei der Hitzesterilisation. In eine der beiden Öffnungen am Grunde des Vorratsgefässes wird dann ein einfaches, 70 mm langes Stahlröhrchen mit leicht konischem Ansatz hineingesteckt und das Vorratsgefäss mit einem Glasüberwurf zugedeckt.

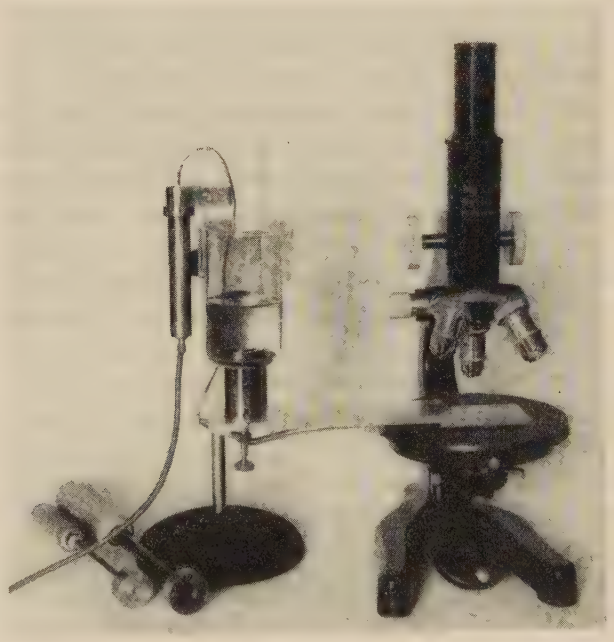

Abb. 2. Die Durchströmungseinrichtung neben dem Mikroskop zur Beobachtung aufgestellt. Am Stativ liegen der Schlüssel für die Haltemutter des Vorratsgefässes sowie der Messingstempel zum Anparaffinieren der Deckgläser auf die Kammer.

Als nächstes werden die beiden zum oberen und unteren Verschluss der Durchströmungskammer notwendigen sorgfältig gereinigten Deckgläser an Ort und Stelle gebracht und mit zwei einfachen Klammern dort festgehalten. Die Kammer ist also dergestalt oben und unten verschlossen, nur dass die Deckgläser noch nicht festparaffiniert sind. Der Gasfilter wird schliesslich mit Watte gefüllt und die Apparatur nach diesen Vorbereitungen trocken sterilisiert.

Am besten, wenn sie noch etwa 80° C warm ist, entnimmt man sie dem Sterilisationsapparat und befestigt sie an einem Stativ. Nimmt man nun mit einer spitzen Pinzette etwas festes Paraffin und bringt es an den Rand des oberen Deckglases, so schmilzt dieses und fliesst in einer sehr dünnen Schicht zwischen Deckglas und Oberseite der Kammer. Auf diese Weise erzielt man mühelos einen dauerhaften und sauberen Verschluss der Kammeroberseite. Nach dem Abkühlen entfernt man die das Deckglas haltende Klammer und dreht die ganze Apparatur an dem Kugelgelenk einmal ganz herum. Vorher muss man dafür Sorge getragen haben, dass der Deckel des Vorratsgefässes beim Umdrehen nicht herunterfällt. Die Kammerunterseite mit dem noch losen Deckglas zeigt dann also nach oben. Man entfernt das Deckglas und bringt nun das zu untersuchende Gewebe in die Kammer ein. Wenn man die Apparatur später wieder in die richtige Lage dreht, hängt das eingebrachte Gewebe also direkt an der Unterseite des oberen Deckglases. Selbstverständlich können bei dieser Anordnung nur Gewebe oder Zellen verwendet werden, die sich auf der Glasunterlage festheften bzw. wenigstens zunächst mit etwas Plasma zu befestigen sind. Ist das Gewebe in die Kammer eingebracht, kann man naturgemäss die ganze Kammer nicht erneut erwärmen, um das zweite Deckglas in der gleichen Weise wie das erste anzuparaffinieren. Man bedient sich deshalb am besten eines einfachen Metallstempels, den man anwärmt und auf das Deckglas kurz aufsetzen kann. Hat man vorher vorsichtig eine kleine Menge Paraffin unter und an den Rand des Deckglases gebracht, so schmilzt dieses schnell und dichtet nach dem Erkalten ebenfalls die Kammer ab.

Sichert man die Deckgläser zusätzlich gegen ein Abspringen mit vier einfachen Klammern, so ist dieser einfache Verschluss der Kammer auch bei 37° C ausreichend dicht und zuverlässig. An Stelle von Paraffin lassen sich selbstverständlich auch andere ungiftige Materialien zum Ankleben der Deckgläser verwenden.

Will man sich eines umgekehrten Mikroskops bedienen, wobei das Gewebe am Boden der Kammer liegen darf, muss man sinngemäss die Kammer erst von unten verschliessen, das Gewebe einsetzen und zuletzt den oberen Verschluss anbringen.

Hat man die Apparatur wieder herumgedreht, kann man das flüssige Medium einfüllen. Je nach Art des benutzten Gewebes ist es nötig hiermit so lange zu warten, bis dieses so fest auf seiner Unterlage haftet, dass es von der strömenden Nährflüssigkeit nicht mehr abgelöst werden kann. Man neigt zum Füllen die Apparatur am besten etwas schräg mit der Kammer nach unten, damit keine Luftblasen in dieser zurückbleiben. Die Flüssigkeit wird einfach in das Vorratsgefäss hineingeschüttet. Sie fliesst dann durch die eine Öffnung am Boden durch die Kammer und schliesslich in der zweiten Leitung wieder zurück. Die von ihr verdrängte Luft kann durch das eingesteckte Röhrchen ungestört entweichen. Nach dem ordnungsgemässen Füllen wird das Röhrchen entfernt und die Gasleitung angeschlossen. Zur Erzielung einer entsprechenden Wasserdampfspannung leitet man das einströmende Gas vorher durch eine Waschflasche. Mit Hilfe des Nadelventils stellt man dann die durchströmende Gasmenge so ein, dass ein langsamer Flüssigkeitsstrom in der Kammer entsteht. Der Wechsel der Nährflüssigkeit geschieht durch einfaches Absaugen und erneutes Füllen des Vorratsgefässes. Will man die Nährflüssigkeit nur einmal durch die Kammer fliessen lassen, so lässt sich das Gasventil als Abflussleitung benutzen. Man verschliesst die Öffnung, aus der sonst oben im Vorratsgefäss das Gas austritt, und dreht das Ventil etwas auf. Die Flüssigkeit tropft dann, je nach der Einstellung, schneller oder langsamer an der Ventilschraube herunter in ein untergestelltes Gefäss.

Die Apparatur wird mit Absicht in dieser allgemeinen und undifferenzierten Ausführung beschrieben, da anzunehmen ist, dass die verschiedenen biologischen Fragestellungen, die mit ihr bearbeitet werden können, entsprechende Änderungen oder Zusätze erfordern. So lässt sich das Vorratsgefäss modifizieren, vergrössern oder mit Messelementen versehen, ebenso wie die Kammer anders zu gestalten ist, auch in der Weise, dass mehrere hintereinander geschaltet werden können. Das Vorratsgefäss sowie andere Teile lassen sich ferner mit einem Heizmantel umgeben, so dass unter gleichzeitiger Verwendung eines Heiztisches die Apparatur für Warmblütergewebe auch ausserhalb eines Thermostaten oder eines entsprechend geheizten Raumes zu benutzen ist.

ZUSAMMENFASSUNG

Die beschriebene Einrichtung zur Züchtung von Geweben im strömenden Medium vereint neben den allgemeinen Vorteilen dieses Prinzips beste optische Bedingungen zur mikroskopischen Beobachtung der kultivierten Gewebe.

Mit Hilfe eines in geeigneter Weise einströmenden Gases wird die Nährflüssigkeit im Kreislauf von einem Vorratsgefäss durch die Beobachtungskammer und wieder zurückgeführt. Im Gegensatz zu früheren ähnlichen Einrichtungen wird an Stelle von Glas nichtrostender Stahl für alle mit der Nährflüssigkeit in Berührung kommende Teile verwandt.

Die Fertigstellung der Arbeit wurde von Herrn Prof. A. Fischer mit Mitteln des National Cancer Institute of the National Institutes of Health, Public Health Service, U.S.A., unterstützt.

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STRUCTURE OF THE MITOCHONDRIA IN THE MALE GERM CELLS OF *HELIX* AS REVEALED BY THE ELECTRON MICROSCOPE

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Received May 21, 1953

The development of a technique suitable for chemical analysis of the mitochondria by Bensley and Hoerr (1) and Claude (2) paved the way for a relatively concentrated attack upon the chemistry and physiology of these structures (6, 13). As a result of these studies, the mitochondria have been found to "... house all the principle oxidative enzymes of the animal cell" (5). In addition, research on the chemistry and physiology of the mitochondria has posed a number of interesting questions bearing on the possible relationship of their function to that of their ultramicroscopic structure; for example, do mitochondria possess physiologically active membranes? Are they little homogeneous corpuscle-like cellules with the enzymes dissolved within them; or do they possess an organized cyto-architecture of a type favorable for enzyme localization and function?

Efforts to answer questions such as these concerning the structure of mitochondria are being made by aid of the electron microscope. Porter, Claude, and Fullam (12) described masses of variable size and relatively high density along the body of the mitochondria in tissue culture cells. Mitochondria isolated from the cell contained granules of the order of 80 to 100 mμ in diameter (3, also 9). Pease and Baker (11) found considerable variation in the structure of the mitochondria within the kidney tubules, presumably associated with the functional activity of the cells. The mitochondria may appear vacuolated, or to possess particles held together in a lattice framework. They also observed a definite membrane surrounding the mitochondria, as had been previously reported (4). According to Palade (10), Watson (14) found "regular laminated membrane structure with the lamina running perpendicular to the axis in the elongated mitochondria". Similar structures have been described (10) in mitochondria from cells of different tissues as well as from those of different animal species. They have been interpreted

as ". . . a system of parallel, regularly spaced ridges that protrude from the inside surface of the membrane toward the interior".

The structure of the mitochondria in male germ cells of *Helix* seems to differ sufficiently from previous reports to warrant a description of it here.

MATERIAL AND METHODS

Pieces of the gonads of *Helix aspersa* were fixed in 2 per cent osmic acid and in Champy's solution. They were embedded and sectioned at approximately 0.1 μ after the method of Massey (7). Electron micrographs were made by use of an RCA model EMU microscope.

DESCRIPTION

The mitochondria in the male germ cells of *Helix* are filaments that measure about 0.217 to 0.304 μ in diameter. A relatively long longitudinal section through a mitochondrium at different levels is shown in Fig. 4 a. It will be noted that all the mitochondria, cut through the longitudinal axis, display a filamentous structure (Figs. 1–7). The filaments, where clearly revealed, are 8 in number, relatively dense, measure about 400 Å in diameter, and seem to be of indefinite length (Figs. 1 a and 1 b). Their arrangement within the mitochondria is more or less parallel, and they do not show evidence of anastomosis. The filaments may appear to be beaded or to display thickenings along their longitudinal axes (Fig. 1 b). Sections in the long axis through the ends of mitochondria reveal the filaments from one side to be continuous as loops with those arranged symmetrically in the other side (Figs. 1 a and 1 b). This suggests that the filaments are really sections through longitudinal coaxial lamellae that are continuous throughout the body and at the ends of the mitochondria. Such an interpretation is in harmony with the structure seen in cross sections of certain mitochondria (Figs. 6 a; 7 a). Here are observed 4 dense circles or concentric lamellae that measure about 400 Å in thickness. In the middle of the cross sections (Fig. 7 a) and in the longitudinal section (Fig. 1 b) is seen a region void of obvious structure. This region, together with that between the lamellae, probably constitutes the matrix.

Examination of the various figures will reveal mitochondria cut at different angles from those described above. A longitudinal section through the lower part of a mitochondrium, showing only the outside lamella as two apparent filaments, is seen in Fig. 7 b. Sections through a higher plane may show only two lamellae in the form of 4 apparent filaments (Fig. 7 c), while sections through the axis of the mitochondria display 8 filaments that represent

sections through 4 lamellae (Fig. 1 b). Oblique sections may give the appearance of filaments extending across the short axis, an arrangement in harmony with the view that they are cut lamellae. The outer lamella constitutes the limiting membrane of the mitochondria (Figs. 1 c, 4 a, 4 b, 5 a, 6 a).

Fig. 2 is a section through a portion of a developing sperm. A transverse section of the axial filament showing its ultramicroscopic components is seen at *a*. The other bodies in Fig. 2 are filamentous mitochondria. Fig. 3 is probably also a portion of a developing sperm. The upper body (*a*) appears to be a section through an axial filament in its initial stage of development. The median and the lower body, also labeled *a*, are mitochondria.

We have attempted to schematize pictorially in Fig. 8 the following interpretation of mitochondrial structure, emphasizing, however, that the interpretation is to be considered as a hypothesis, and, if true, may not necessarily hold for mitochondria in all the cells of other species: The mitochondria appear composed of a series of 4 coaxial longitudinally arranged lamellae of about 400 Å in thickness. These lamellae are continuous at the ends of the mitochondria in the form of a dome. The regions at the center and between the lamellae are presumably filled with a matrix void of obvious structure. This conception of the mitochondria might be roughly compared to a series of 4 gelatin capsules of graduated size, placed one within the other. The space within the middle capsule and that between the walls of the succeeding capsules presumably contain the matrix, the outer capsule being comparable to the mitochondrial membrane. The nature of the framework maintaining the lamellae is not clear. However, the thickenings on the longitudinally cut lamellae might conceivably represent attachments for perpendicularly arranged lamellae or ridges extending inward from each lamellar surface. This view is only conjecture and lacks substantial evidence to support it. However, such an arrangement of ridges would be somewhat similar to that proposed by Palade (10) for the mitochondrial membrane.

DISCUSSION

The structure here reported for *Helix* mitochondria is in accordance with the observations of Monné (8) who states that "Short rod-shaped and long filamentous mitochondria are positively birefringent in longitudinal direction. This birefringence is due to the protein component of the mitochondria." Why there should be a difference in structure of the mitochondria in *Helix* male germ cells from that reported in other cells is not clear. However, it may be that further investigation will establish the difference as due to a

variation in structure within the mitochondria of certain animal species. This perhaps is not unreasonable to suppose since mitochondria are known to differ greatly in size, shape, number and even chemistry in different kinds of cells.

We do not think that the structure described here in the mitochondria is due to artefact. However, if it is an artefact, it is a consistent one, since we have seen it in the mitochondria of over 100 cells. No one can say how much is real and how much is artefact in preparations of this kind, because at present there is no way of observing structural detail in living mitochondria. However, it is apparent that the methods of fixing used reveal the ultramicroscopic structure of the developing sperm tail filament.

No attempt is made to correlate the structure with the function of the mitochondria, save for the suggestion that such a structure would provide added intramitochondrial surface and compartments that are conceivably favorable for enzymatic function.

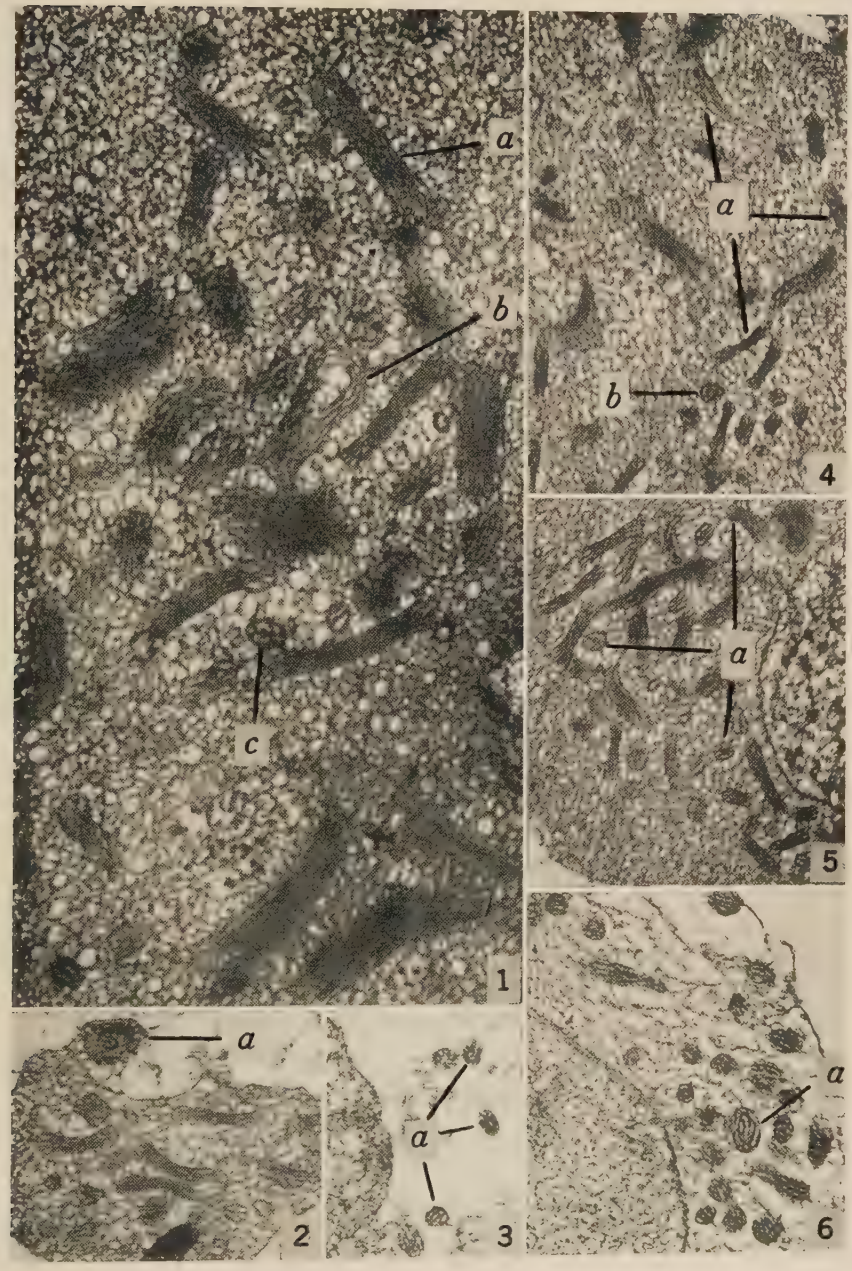
SUMMARY

The mitochondria of the male germ cells of *Helix* reveal longitudinally arranged filament-like structures. There are often 8 filaments that measure about 400 Å in thickness in each mitochondrium. These filaments are interpreted as sections of 4 longitudinally arranged coaxial lamellae, that are continuous at the ends of the mitochondria in the form of dome-shaped structures. The regions at the center and between the lamellae are thought to constitute the mitochondrial matrix.

We wish to thank Mr. L. Evans Roth and Mrs. Vera A. Johnson of the Argonne National Laboratory for kindly taking the electron micrographs for us, and Mr. William J. Ketcham for preparing the stereogram. Thanks are also due to Marietta Issidorides of the State University of Iowa for aid in the preparation of the manuscript.

All figures are sections through portions of the male germ cells of *Helix*. Fixation 2 per cent osmic acid.

Fig. 1. Mitochondria cut at different planes and angles showing their filamentous structure. Sections through ends of mitochondria are seen at (*a*) and (*b*). 20,000 ×.
Fig. 2. Section through spermatid revealing cross section of developing tail filament (*a*). Other bodies are filamentous mitochondria. 10,000 ×.
Fig. 3. Upper body (*a*) is probably through a young developing sperm tail filament. Other bodies are mitochondria. 10,000 ×.
Figs. 4 and 5. Sections through mitochondria at various planes and angles illustrating their filamentous structure. 10,000 ×.
Fig. 6. Thin section at (*a*), shows slightly oblique cut filaments. 10,500 ×.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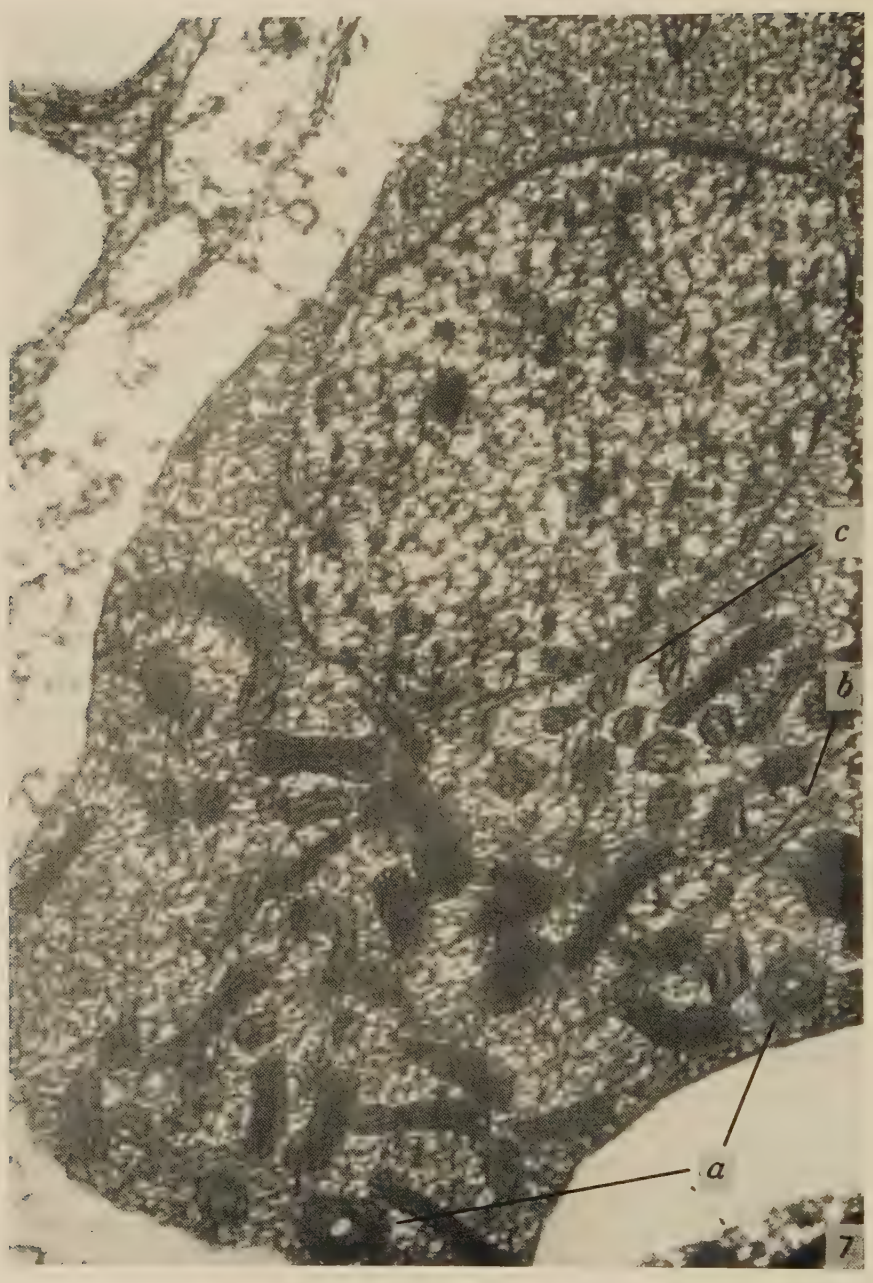

Fig. 7. Slightly thicker section showing depth and filaments within the mitochondria. Note cross sections of filaments (*a*). 20,000 ×.

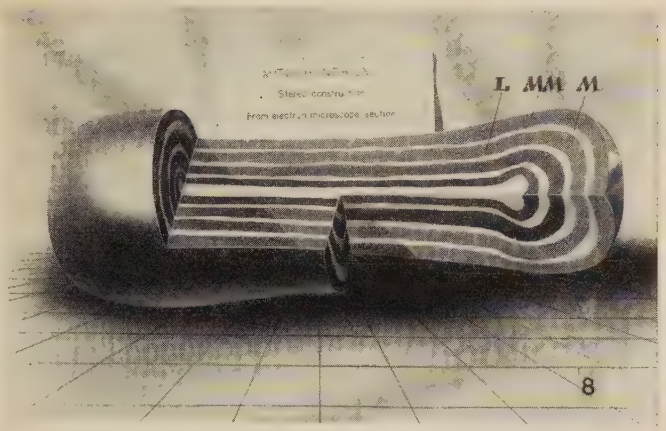

Fig. 8. Schematic stereogram illustrating the proposed mitochondrial structure in *Helix* male germ cells. A lamella is shown at *L*, matrix at *M*, and outer lamella or membrane at *M M*.

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A NEW TRANSFORMING AGENT DETERMINING PATTERN OF METABOLISM OF GLUCOSE AND LACTIC ACID IN *PNEUMOCOCCUS*[1]

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Received May 22, 1953

The starting point of the present investigation was the observation that the Type III encapsulated pneumococci arising from transformations induced in certain unencapsulated strains are of two sorts: the majority give rise to colonies having diameters approximately 3 times larger than rough colonies have, while a few form colonies which are 4 times larger. Clones set up from the two sorts maintain indefinitely, under appropriate conditions, their characteristic difference in colony size. The experiments to be described below are concerned with the origin and nature of the pneumococci which give rise to large colonies (LC pneumococci).

The interest of the observation emerges from the following considerations. In the past it has been noted that the size of a pneumococcal colony is often correlated with the amount of capsular polysaccharide secreted (8, 9, 14). Thus, the finding of the LC clones raised the problem, already considered in an earlier publication (15), of whether the Type III capsular agent acts as a discrete unit, or whether, on the contrary, it induces the formation of a spectrum of transformed bacteria exhibiting varying degrees of polysaccharide secretion. If the latter were true, LC clones might be pneumococci which secrete very large amounts of capsular polysaccharide, and belong thus in the upper limits of such a spectrum. In the earlier study it was concluded that the capsular transforming agent acts in an all-or-none fashion. The present study confirms this conclusion, for it was found that the colonies of the transformed Type III pneumococci form two discrete distributions with respect to diameter. No evidence for a spectrum could be obtained.

The experiments to be described show that LC pneumococci are produced by a double transformation, in the course of which a bacterium acquires not only the capsular agent, but also a hitherto unrecognised agent, present in extracts prepared from the Type III strain usually employed in capsular transformation studies. The new agent (LC agent) is apparently independent

[1] A grant to the Faculté des Sciences from the Damon Runyon Fund for Cancer Research is gratefully acknowledged.

of the capsular agent, both as a genetic entity and with respect to its physiological activities, for it can be acquired singly by unencapsulated bacteria, producing in them a change which results in their forming colonies which are slightly larger and distinctly more opaque than those of ordinary unencapsulated clones. A study of the metabolic differences between normal and LC strains will be described. The results of this study demonstrate that LC strains have a distinctive pattern of glucose metabolism, and that they fail to oxidize lactic acid.

The present report is subdivided into two chapters, one dealing with biometric and transformation studies, the other describing metabolic studies.

I. BIOMETRIC AND TRANSFORMATION STUDIES

Two questions were investigated: 1) Are capsular transformations induced in all-or-none fashion? and 2) What is the origin of the LC pneumococci? These problems were investigated by the measurement of many colonies of encapsulated pneumococci, obtained by spreading on petri dishes populations of unencapsulated pneumococci in which transformations had been induced by treatment with various specific transforming extracts.

MATERIAL AND METHODS

The methods of culture, preparation of transforming agents, and induction of transformations were those described in a previous publication (4). The only modification introduced was the more careful standardization of the blood-agar plates on which the colonies to be measured were grown. The important factors to control are glucose content and surface humidity. With respect to the former, 2.0 cc of a sterile 2.5 per cent glucose solution were added for every 150 cc of agar medium. Surface humidity was reduced by drying the plates overnight at 37° C. In any single experiment the same volume of culture, or dilution thereof, was spread on all of the plates. In the early part of the work, sterile defibrinated rabbit blood was employed in the agar medium, while later horse blood was used. Mr. Lemetayer, Director of the Institut Pasteur at Garches, and Mr. Girard, Chef des Services de Saignées, very kindly supplied horseblood in the large quantities which proved necessary for completing the present study.

Dilutions of cultures for plating were made in 2 per cent Difco Neopeptone dissolved in 0.85 per cent NaCl.

Even with above precautions, some variation was observed in the mean diameter of colonies of a given strain when plated on different preparations of medium. Consequently, direct comparison of various experiments was not always possible. Duplicate plates gave, however, very consistent results. All measurements of colonies were performed after 17 hours of incubation at 37° C. Longer incubation produces larger colonies, but autolysis becomes very pronounced when incubation is continued

beyond the chosen time interval. Measurements were carried out with a Zeiss ocular screw-micrometer mounted on a binocular dissection microscope The overall magnification was 9 fold. The petri dishes were held on an inclined stage, and their surface illuminated obliquely. With an even illumination, the colony margins were very sharp. The diameter of a colony was measured at right-angles to the direction of the inclination.

The pneumococcal strains which were transformed were R36A, the rough strain used in the classical experiments of Avery, MacLeod and McCarty (2), and SIII-1-T50, an intermediate smooth strain produced by transformation of the above with an extract obtained from the mutant Type III strain SIII-1 (15).

At the outset, transforming extracts were prepared from the Type III strain A66. This strain contains the normal, or SIII-N capsular agent, and forms very large amounts of polysaccharide. As the present study evolved, transforming extracts were prepared from various clones isolated from transformation experiments. These will be described in the course of presenting the results.

EXPERIMENTAL RESULTS

1. *The diameters of SIII-N colonies of independent origin.* If the capsular agent induces transformations in an all-or-none fashion, independently transformed bacteria should be identical with respect to capsule. A definitive study of this point is not possible, owing to lack of quantitative and qualitative methods of studying polysaccharide secretion in a large sample of clones set up from such bacteria. However, a procedure was developed which, with certain reserves, can be considered to serve this purpose. Differences in the amounts of polysaccharide secreted are often reflected in differences in the diameters of colonies: the more polysaccharide, the larger the colony. Thus, measurements were made on many colonies derived from Type III pneumococci which had arisen independently of each other by transformation of an unencapsulated strain. The characteristics of the distribution obtained were compared with those of a second distribution, obtained from measurements of colonies derived from the progeny of a single Type III pneumococcus, also made by transformation. This second distribution is a control, describing the variability of the diameters attained by colonies grown from sister cells. If the capsular agent acts in an all-or-none fashion, the characteristics of these two sorts of distributions should be identical, for the variability in both instances should be due only to normal variation in the growth on plates.

The first, or experimental distribution, was obtained in the following way. The strain undergoing transformation was treated with the Type III agent under optimal transformation conditions. After incubation, dilutions of the treated cultures were spread on blood-agar plates. All colonies which were

more mucoid than the inoculated strain were measured, provided they did not fall in a crowded area. At least four treated cultures were employed for establishing a single distribution. Thus, while some of the mucoid colonies on a plate made from a given culture might be the progeny of a single transformed bacterium, the colonies measured for a single distribution cannot be derived from less than four independent transformation events. It is indeed probable that they are derived from very many more.

The addition of as little as 0.005 micrograms of DNA, isolated from an SIII-N strain, to an appropriate medium containing transformable pneumococci suffices to induce the transformation of a few bacteria. It has been found that the addition of 1000 times this amount of nucleic acid induces transformation of about 0.5 per cent of the unencapsulated bacteria during a time interval of 30 minutes, provided the bacteria are in the state which permits them to react with the nucleic acid (10). Under the conditions of the present experiments, the transforming agent is present in about 1000 fold excess of the minimal amount required to obtain transformation. At the time when the bacteria become capable of reacting with the agent, their population density is 16 000 per cc. The reactive state persists for two hours. Assuming an incidence of transformation of 0.5 per cent which is a minimum estimate, the encapsulated bacteria appearing at the end of the incubation period normally given to transformation cultures (17–20 hours) would on the average derive from 16×5, or 80, transformations in each of the treated cultures.

In the earlier experiments, treated and control cultures were incubated 18–20 hours prior to spreading on plates. However, it was found that this long incubation led to a wide spread in the measured diameters, owing, no doubt, to the onset of adverse conditions after the cessation of growth in the transformation medium. In later experiments, platings were made after only 12–14 hours of incubation. Nonetheless, the distributions of colony diameters obtained from transformation experiments are somewhat wider than those observed when the same bacteria are taken from blood-broth medium.

The clone utilized for preparing the control distribution was derived from a single Type III colony isolated from a previous transformation of the same strain upon which the experimental distribution was to be obtained. Control clones were grown under the same conditions as those employed for inducing transformations, in order to eliminate differences in the distributions which might be due to or result from differences in cultural conditions prior to plating.

The results of a typical experiment are shown in Fig. 1. In this particular instance, the strain undergoing transformation was SIII-1-T50. As has been already stated, the control clone came from a single SIII-N colony recovered

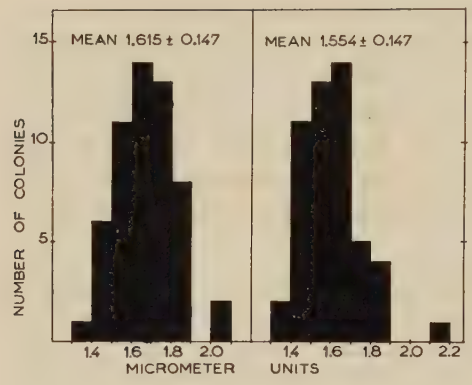


Fig. 1. Histograms constructed from the measurements of diameters of colonies of encapsulated Type III pneumococci. On the left, the bacteria came from four cultures in which multiple transformations had been induced. On the right, the bacteria came from a clone isolated a few days previously from a transformation experiment. The strain undergoing transformation was SIII-1-T50. Both the means and the standard deviations are essentially the same.

from a transformation of strain SIII-1-T50 a few days earlier. It can be seen that the distributions of the diameters of the SIII-N colonies are essentially the same whether the bacteria come from the control clone, or directly from a transformation culture. It is clear, therefore, that there is no obvious difference between the various SIII-N colonies which arise from the control clone, and those which arise from different transformed SIII-1 pneumococci. Thus, the SIII-N agent does not appear to give rise to a spectrum of pneumococci having graded polysaccharide secretion, but on the contrary seems to behave as a discrete entity.

If now new clones are set up, selecting the largest and smallest of the colonies of a particular distribution, usually, all of these clones give rise to essentially identical distributions, when the colonies formed by them are measured, and these distributions do not differ from the original one. However, the following exception to this rule is observed: from time to time, when a clone is set up from a very large colony, it gives rise to colonies which are on the average about 25 per cent larger in diameter than the majority of SIII-N colonies measured. These constitute the LC clones.

The appearance of these clones in the transformation cultures is very rare. Often, none are found. However, in one out of ten distributions obtained from transformed cultures of strain SIII-1-T50, a definite bimodality was found. This aberrant distribution is shown in Fig. 2. The second mode was found to be due to the presence of an unusually large number of LC clones in this particular experiment.

Attempts were made to determine the average number of cells per colony in the two kinds of clones. Several well isolated colonies from each were homogenized in a measured volume of medium, and serial dilutions of the suspensions plated. It was found that whereas the results were concordant when the colony came from an SIII-N-

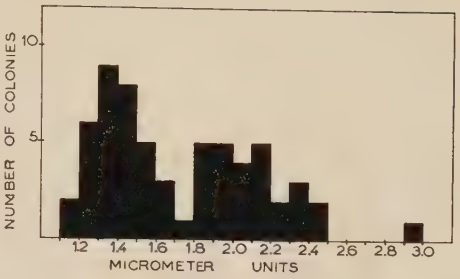


Fig. 2. Histogram constructed from measurements of encapsulated Type III colonies grown from bacteria of four cultures in which multiple transformations had been induced. Out of ten sets of such measurements, only this one showed bimodality. The peak on the right is caused by the presence of an exceptionally large number of large colonies in this particular experiment. The peak on the left corresponds to the single peak in Figure 1.

LC clone, they were aberrant when the colony was SIII-N. The aberration consisted of an excess of colonies in the higher dilutions. An experiment was set up to see whether concordant colony counts would not be obtained if the initial suspension were incubated a short time before diluting and plating. This was tried, leaving the suspensions 15 minutes at 37° C, and one hour in the refrigerator before diluting and spreading on plates. The counts on serial dilutions were then in agreement, in both types of clones. Furthermore, while the average number of cells per colony of SIII-N-LC remained unchanged by this treatment, the number of cells per SIII-N colony was increased by 30 per cent. The ratio of these averages was 3:1. The experiments are of interest in that they suggest that SIII-N colonies may be smaller because these bacteria have a lowered viability under aerobic conditions, undergoing autolysis more readily.

From these experiments it may be concluded that the SIII-N pneumococci produced by the action of the SIII-N transforming agent on strains R36A and SIII-1-T50 fall into one of two categories of which the first is more frequent: clones which form colonies of the diameter shown in Figure 1, and clones characterized by unusually large colonies. In Fig. 3 are shown the relative diameters of normal and LC clones. The data for this figure were obtained by measuring the colonies produced by one LC clone and one normal clone, grown under identical conditions and measured the same day. Fig. 3 also includes measurements made the same day on strain SIII-2, an intermediate smooth Type III clone which has been mentioned very frequently in previous publications (4, 13, 15). The three clones are quite distinct.

In the course of this study, a surprising mutability was observed in the clones derived from the transformed bacteria. In one experiment, after 3 transfers of 13 clones, two contained mutant forms which were less mucoid than SIII-N pneumococci. In another experiment, similar mutations were observed in 3 out of 8 clones after growth of the first transfers. This may be due to a real mutability of the capsular agent itself in these transformed clones. On the other hand, it may be that unencapsulated strains tend to evolve toward a state in which capsular synthesis is disadvantageous, so that endowing them with capsules by transformation upsets the pattern of selection. Under these circumstances, mutations of the capsular agent,

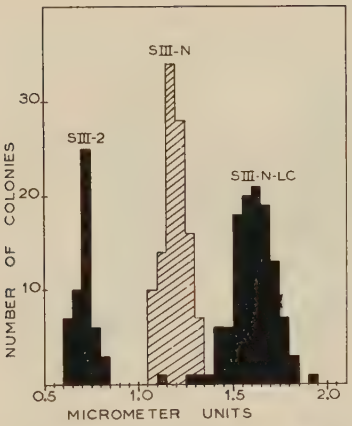


Fig. 3. Histograms constructed from measurements of colonies grown from three different clones of pneumococci. Clones SIII-N and SIII-N-LC were isolated from single colonies, the measurements of which are included in Fig. 2. Clone SIII-N came from a colony in the left hand distribution, while clone SIII-N-LC from a colony in the right hand one. For comparison, colonies of mutant Type III strain SIII-2 were measured simultaneously and included in the diagram.

leading to diminished capsule synthesis, would be favored, as would also be favored mutations reversing the trend established by prolonged existence in the unencapsulated state.

2. *The homogeneity of type III strain A66.* Since the transforming extract of strain A66 obviously induces the formation of two kinds of Type III pneumococci, it becomes essential to know whether strain A66 is heterogeneous, being composed of these two cell-types, or whether, on the contrary, both activities reside in the nucleic acid fraction of one cell-type. Therefore, measurements were made upon colonies of the stock strain A66. On the same day, and on the same media, measurements were also made on colonies of a clone of SIII-N pneumococci, isolated from a previous transformation experiment. The results of these measurements are shown in Figure 4. It is clear from these measurements that the colonies of strain A66 have a mean diameter about 20 per cent larger than that of the SIII-N colonies of the control clone. Thus, strain A66 is best described as an SIII-N-LC clone. There is no indication that the smaller colony type is present in the population A66 in any appreciable numbers.

This being so, the simplest working hypothesis consists of supposing that both colony types arise from the activities of the nucleic acid isolated from an essentially homogeneous population, and that the two activities are due to two factors which reside in each pneumococcus in the A66 population. One of these factors is the capsular agent, while the other is presumably a new agent, responsible for the large colony size. The rareness with which SIII-N-LC pneumococci are formed by transformation would thus be the consequence of the rareness of two different transformations taking place in the same bacterium.

Fig. 4. Histograms constructed from measurements of colonies derived from strain A66, on the right, and an SIII-N clone on the left. The SIII-N clone was made by transformation of an SIII-1 bacterium by an extract of strain A66.

3. *The capsular agents present in SIII-N and SIII-N-LC strains.* If the above hypothesis is correct, the capsular agents in both types of clones should be the same. To test this point, nucleic acid extracts were prepared from two clones, one SIII-N-LC and one SIII-N (TPs 36E and 30B, respectively). With these extracts, transformations were induced in strains R36A and SIII-1-T50, the treated populations were spread on plates, and the colonies obtained measured in the usual fashion. In a typical experiment, 65 smooth colonies from plates of cultures treated with TP 36E and 96 from plates of cultures treated with TP 30B were measured, yielding the mean values of 2.02 ± 0.173 and 1.95 ± 0.117 micrometer units respectively. The difference between the means is not significant, and it can be concluded, therefore, that both TP preparations induce the formation of essentially the same sort of encapsulated bacteria. Thus, strain SIII-N-LC contains the same capsular agent as strain SIII-N.

However, it was noted in the course of these experiments that in the cultures treated with TP 36E there occasionally appear some SIII-N-LC clones, while none are found in the cultures treated with TP 30B. In its biological activity, TP 36E thus resembles the transforming extracts prepared from strain A66. This finding is entirely in agreement with the hypothesis that the LC trait is determined by an agent similar to the capsular agent, which can be transferred to pneumococci by the transformation technique.

4. *The isolation of unencapsulated LC clones.* If the properties of the LC strains are determined by a transforming agent, and if this agent is indepenent of the capsular agent, then one should expect unencapsulated bacteria to be able to acquire the former without the latter. Indeed, this transformation should occur more frequently than the double transformation in which

both are acquired. Detection of R-LC or SIII-N-LC bacteria would depend upon whether transformation by the LC agent alone suffices to produce a recognizable morphological change.

A careful search among the previously supposed "untransformed" members of treated populations of R and SIII-1 pneumococci revealed that some of the colonies produced by them were, in fact, different from ordinary R or SIII-1 colonies in that they were somewhat larger and very much more opaque. The incidence of the aberrant colonies in the treated cultures was never greater than 0.7 per cent. None were ever found in cultures which had not received a nucleic acid preparation from an LC strain. Clones derived from the aberrant colonies were established, but these could be maintained free of reversions only when carried on solid medium (blood-agar). Measurements of colonies of normal and aberrant clones of strain SIII-1-T50 gave the average values of 0.796 ± 0.143 and 0.983 ± 0.192 micrometer units. The differences between the means is not significant, and, indeed, on the basis of size alone it would be difficult to ascertain that two distinct colony types exist. However, the opacity of the aberrant colonies permits an almost certain differentiation when the plates are examined in transmitted light.

Since it was suspected that these aberrant colonies might be derived from pneumococci which had acquired the hypothetical LC agent but not the SIII-N agent, the next step was to transform them with the capsular agent, to see whether or not they yielded SIII-N-LC bacteria. Just prior to subjecting various clones to transformation, they were transferred from plates into liquid medium, using large inocula. These cultures served to inoculate transformation cultures and were discarded afterwards. In the particular experiments to be described, the aberrant clones were transformed with a nucleic acid extract of strain A66. At the same time, controls were made by transforming normal strains with the same nucleic acid preparation. The treated populations were streaked on sectors of agar medium plates, rather than being spread for measurement. From each treated population, one transformed colony was chosen at random, grown out in blood-broth medium, and samples of the resulting culture plated for measurement: Quadruplicate transformation cultures were made of each aberrant clone and of each control strain. Thus, for each strain treated with the nucleic acid fraction of strain A66, the progenies of four independently transformed bacteria were measured. This method of study was chosen to avoid the greater variability in colony diameter which is observed when measurements are made directly on the colonies of the transformation cultures themselves. The results of some measurements are shown in Fig. 5. From these observations, it is clear that when

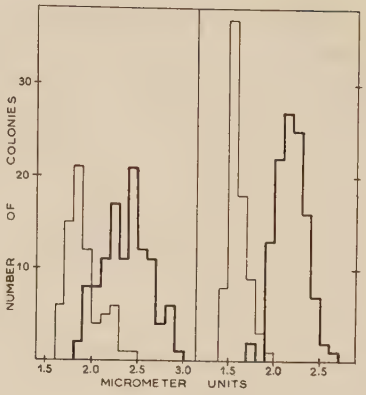


Fig. 5. Histograms constructed from measurements of SIII-N colonies derived from transformation of normal and aberrant clones of strains SIII-1-T50 and R36A. For the pair of curves on the left, the left-hand distribution came from measurements of colonies of transformed normal SIII-1 pneumococci, while the distribution on the right came from measurements of transformed aberrant SIII-1 pneumococci. The pair of distribution curves to the right are the results of similar measurements on transformed normal and aberrant R36A. In both cases, the transformed aberrant clones gave rise to larger colonies on the average than did the transformed normal clones.

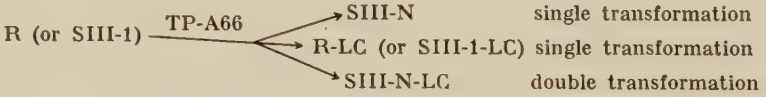
the clone undergoing transformation is an aberrant clone, be it R or SIII-1, the SIII-N bacteria induced are all SIII-N-LC; that is, they form colonies with a mean diameter roughly 25 per cent larger than that of colonies of the majority of the transformed bacteria arising in the normal strains of R or SIII-1 pneumococci. This result is entirely consistent with the hypothesis that the aberrant R and SIII-1 strains already contain the postulated LC agent, which they had acquired independently of the capsular agent in a previous transformation.

The aberrant R and SIII-1 clones will thus be referred to as R-LC and SIII-1-LC clones.

5. *A quantitative study demonstrating that the LC agent is a specific component of nucleic acid extract 36E.* Pneumococci exhibiting the LC phenotype were found only when R or SIII-1 bacteria were treated with a nucleic acid fraction prepared from strain A66, or from an SIII-N-LC strain recovered from a transformation experiment. Since in general the incidence of LC clones is low in a transformed population, it was conceivable that failure to find such clones after treatment with other nucleic acids might be due to sampling error. Accordingly, an experiment was performed in which populations of strain R36A were treated with nucleic acid extracts 36E and 30B, the former obtained from an SIII-N-LC strain and the latter from SIII-N, the incidence of R-LC colonies was scored, and the data analysed statistically. A total of 11 transformation cultures were studied, 4 receiving TP-36E and 7 receiving TP-30B. Samples of the treated cultures were spread on plates, and after incubation, the total number of colonies and the number of LC colonies per plate were estimated from counts. A large number of colonies from the seven cultures treated with TP 30B were carefully examined. After

searching for LC colonies by holding the plates against a light source, they were examined under a binocular microscope. Whereas ten LC colonies were found on the plates made from the four cultures treated with TP-36E, none were found on those made from the seven cultures treated with TP-30B (see Table I). In view of the large number of colonies examined, it is very unlikely that the observed difference is due to sampling. The experiment thus confirms the hypothesis that TP-36E contains a specific agent responsible for the appearance of the LC bacteria in the treated populations and that this agent is absent from TP-30B.

All of the biometric and transformation studies agree with the hypothesis that the nucleic acid fractions of strain A66 and of SIII-N-LC strains contain a hitherto unrecognized transforming agent which is responsible for the LC phenotype. The presence of this agent in strain A66 accounts for the size difference observed between colonies of this strain and the majority of colonies arising from transformations induced with nucleic acid derived from it, for the two transformable strains most frequently used in transformation experiments differ from strain A66 by at least two factors: the capsular agent and the LC agent. Transformations induced by nucleic acid extracts prepared either from strain A66 or from SIII-N-LC strains produced by transformation thus consist of the following inductions:



II. METABOLIC STUDIES

The inconvenience of colony diameter as a criterion for the identification of clones is that it lacks specificity. Thus, the homogeneity of the class of strains which have been called LC remained doubtful until a more specific criterion for identifying them was found. The metabolic studies to be presented below provided such a criterion.

The observed differences in colony size are manifested on blood agar plates; that is, under aerobic conditions and where glucose limits the amount of growth. A metabolic difference was therefore sought in the aerobic metabolism of glucose by normal and LC strains. In addition, a second possible difference was sought, since it had been noticed that the two types of colonies produced different degrees of greening of the blood. Since greening is due to the liberation of H_2O_2 by the bacteria, manometric experiments on glucose

oxidation were set up in such a way as to permit a simultaneous study of the amount of H_2O_2 formed by each of the strains.

No systematic study has apparently ever been made of the oxidative metabolism of pneumococci. Finkle (5) observed differences in the Q_{O_2} of three smooth races of different capsular type, as well as in rough races derived from the latter. In phosphate buffer, the optimum O_2 consumption was found to occur at pH 7.8. Observations of Sevag and Maiweg (13) demonstrated that without the addition of protective agents to the bacterial suspensions, respiration was rapidly inhibited by the accumulation of H_2O_2. Catalase and pyruvate were effective in maintaining enzymatic activity, the former by destroying peroxide, the latter by reacting chemically with it.

TP	Colonies examined	LC
36E	4 456	1
	5 352	1
	5 192	2
	8 200	6
Total	23 200	10
30B	12 222	0
	17 208	0
	14 274	0
	7 770	0
	9 705	0
	9 224	0
	11 484	0
Total	81 887	0

←TABLE I. Comparison of the transforming activities of two different transforming extracts, the one prepared from an SIII-N-LC clone (TP-36E) and the other from an SIII-N clone. The strain transformed is R36A. Four cultures were treated with the first TP and seven with the second, and from each culture the indicated number of colonies was examined.

TABLE II. Comparison of an LC and a non-LC strain with respect to ability to oxidize lactate.

Strain	μ moles glucose	μ moles lactate	μ L. O_2 consumed	Moles O_2 / M. glucose
SIII-1	5	—	182	1.58
	—	10	44	
	—	—	5	
SIII-1-LC	10	—	120	0.51
	—	10	3	
	—	—	6	

Catalase added. Duration of expt. 3 hours

MATERIAL AND METHODS

1. *General method.* In order to study glucose oxidation, inactivation of enzymes by peroxide must be avoided. At the same time, to work constantly in the presence of catalase is impractical since it renders impossible study of the formation of H_2O_2. The following method was therefore adopted: thick bacterial suspensions are prepared, and a very limited amount of glucose was added. Respiration was allowed to go to completion. One Warburg vessel received glucose and catalase, and when O_2 consumption fell to the level of endogenous respiration, more glucose was tipped in to verify that the cessation of respiration had not been due to enzyme inactivation. In a second vessel catalase was held in reserve while the glucose was oxidised. When respiration ceased, the catalase was tipped in and the released oxygen measured. Since under these conditions, the difference in respiration in the presence and absence

of catalase was always equal to the amount of oxygen released by catalase in the second vessel, it is assumed that the glucose in the second vessel was completely oxidized, and that a correct evaluation of H_2O_2 production is obtained.

In some experiments, glutathion was employed for study of H_2O_2 production since this substance reacts rapidly with H_2O_2 without producing a change in gas volume (11). Glutathion as well as thioglycollic acid is very effective in protecting the activity of pneumococcal enzymes in the course of glucose oxidation, and the advantage of this medhod is that it makes it certain that oxidation in the absence of catalase has gone to completion. Two experimental vessels are required: one receiving glucose and catalase, the other glucose and glutathion, the difference in the volume changes being equal to the amount of O_2 liberated by catalase in the first vessel. At high pH values, the inconvenience of the method resides in the difficulty of correcting accurately for the autoxidation of the SH compound when it is in excess. In this respect, thioglycollic acid appeared to be the better reagent. However, with strains of bacteria in which peroxide production is known, these substances could be used very advantageously in various metabolic studies, since any appreciable excess of the SH compound could be avoided. All difficulty can be avoided, of course, by working at a pH below 7. It should be noted that the ability of SH compounds to react rapidly with H_2O_2 has been overlooked by bacteriologists and biochemists, who have been inclined to explain the beneficial action of these substances on the growth of certain microaerophilic bacteria as due to a direct action on the SH groups of bacterial enzymes. It is likely that in certain cases their primary activity is simply to destroy hydrogen peroxide.

2. *Pneumococcal strains.* Various LC and non-LC strains were studied. However, the bulk of the work was done on two clones, one normal, and one LC, both of which possessed the mutated capsular transforming agent SIII-1. Since LC strains are stable only on solid medium, both normal and LC clones were carried on agar to avoid any differences in cultivation of the strains to be compared.

3. *Preparation of cell suspensions.* Pneumococci undergo autolysis readily, so that great care must be exercised in growing the bacteria and preparing cell suspensions. All cultures were initiated by transferring several loopfuls of cells from agar petri dish cultures to liquid medium (medium 1, reference 4). After the first appearance of turbidity, the cultures were incubated another 2 hours and placed in the refrigerator for the night. Next morning, after addition of 0.5 cc of a sterile 2.5 per cent glucose solution per 100 cc of culture, the cultures were placed for 1 hour, in a 37° C water bath. Thereafter, the bacteria were harvested by centrifugation, washed once with a small volume of the salt mixture to be described below, and resuspended in the desired volume of this same solution. 200 cc of culture sufficed for a 4 vessel experiment, the washed cells being taken up in 9 cc of salt solution. For larger experiments, larger amounts were prepared in the same way. Suspension density was controlled photometrically. Microscopic examination revealed that suspensions contained short chains of gram positive diplococci, homogeneous in size.

4. *Solutions.* All solutions were prepared in Pyrex distilled water. The suspension medium contained 42 mg of NaCl, 230 mg of KCl, 60 mg of CaCl and 95 mg of $MgSO_4 \cdot 7H_2O$ dissolved in 75 cc of water. To this were added 25 cc of M/7.5 phosphate buffer pH 7.8.

Warburg vessels receiving catalase were given 0.2 cc of a solution of crude catalase powder, 1/1000 (Armour and Co.) prepared fresh for each experiment.

The glucose employed was "Dextrosol" (Société des Produits du Maïs, Paris), and the lactate a racemic preparation (Malinkrodt).

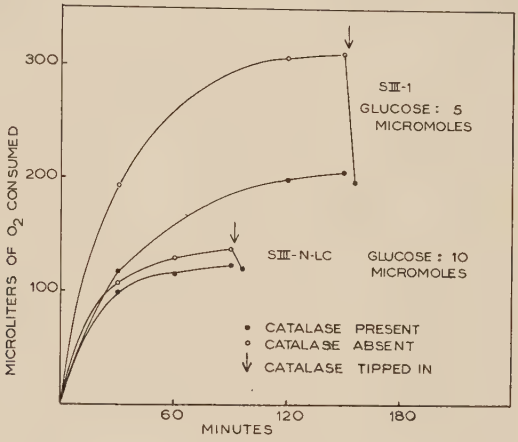


Fig. 6. Results of a typical respiration experiment with an LC strain and a non-LC strain of *Pneumococcus*.

EXPERIMENTAL RESULTS

1. *Respiration and H_2O_2 formation with glucose.* The results of a typical experiment on a normal and an LC strain are shown in Fig. 6. In this particular instance, the non-LC strain contained a mutated capsular agent, while the LC strain contained a normal capsular agent. As will be shown presently, the kind of capsular agent possessed by the bacterium has no influence on its respiration in the presence of glucose. From this figure, two differences between LC and non-LC strains can be seen. First, the LC strain consumes very much less oxygen per molecule of glucose than the non-LC strain. Second, H_2O_2 production by the LC strain is both absolutely and relatively less than that of the non-LC strain.

For purposes of comparison of the respiration of various strains in the presence of the same amounts of glucose, Fig. 7 has been prepared. It summarizes results obtained with three kinds of strains: *a*), non-LC strains, represented by R36A, SIII-1-T50, and an SIII-N strain made by transformation of the latter with the nucleic acid extract of strain A66, and in the course of which only the SIII-N capsular agent was acquired; *b*) an LC strain, made by transformation of strain SIII-1-T50 with the above nucleic acid extract, and as a result of which both the LC agent and the capsular agent were acquired: and *c*), strain A66 itself, the stock laboratory strain which

possesses both the SIII-N capsular agent and the LC agent. For each molecule of glucose, the first group of strains consumes 1.6–1.8 molecules of oxygen, the second strain, about 0.4 molecules, while the last strain consumes 1.1–1.2 molecules. In each instance the cessation of oxygen uptake was due to exhaustion of glucose.

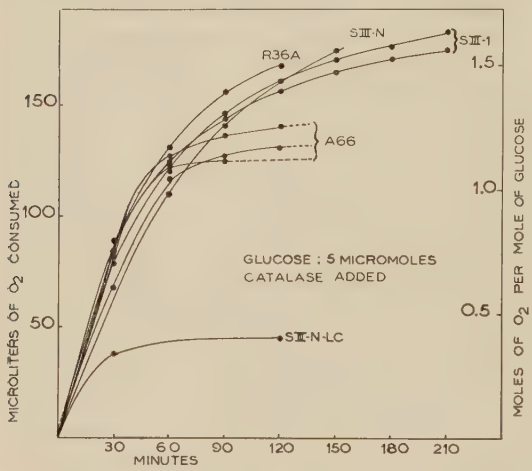


Fig. 7. Respiration of various strains of *Pneumococcus* in the presence of 5 micromoles of glucose. Strains R36A, SIII-1 and SIII-N are non-LC strains; Strain A66 is the naturally occurring Type III strain in which the LC agent was first found; Strain SIII-N-LC is and LC strain made by transformation of strain SIII-1-T50 with an extract from strain A66.

Two points of interest can be noted in Fig. 7. First, particularly evident is the magnitude of the difference between the non-LC strains and the LC strain made by transformation. Second, it is quite clear that although strain A66 has a less active oxygen consumption than the non-LC strains, it is nonetheless more active than the LC strain made by transformation. Thus, although strain SIII-N-LC has acquired two agents from the nucleic acid fraction of strain A66, the two strains are not identical in their metabolic properties. It must be supposed, therefore, that strain A66 differs from strains R36A and SIII-1 not only in possessing the LC and SIII-N agents, but also in an inherited ability to carry out further oxidation of glucose in spite of the LC agent being part of its genetic constitution.

Although strains A66 and SIII-N-LC differ in this respect quantitatively, qualitatively their respiration is very similar. Strain A66 forms very little free hydrogen peroxide, just as strain SIII-N-LC. An even more striking qualitative similarity between strain A66 and LC strains made by transformation will be described in the section below.

2. *Respiration in the presence of other substrates.* Neither LC or non-LC pneumococci, grown as described above, oxidize pyruvate, gluconolactone or 6-phosphogluconate. However, with respect to oxidation of lactate, the

two kinds of pneumococci differ sharply. Whereas lactate is oxidized by strains R36A, SIII-1-T50 and by most encapsulated strains produced by their transformation, this substrate is non oxidized by strain A66, or by any LC strain produced in the transformations described above. Typical experimental results are shown in Table II. Again, it should be emphasized that ability

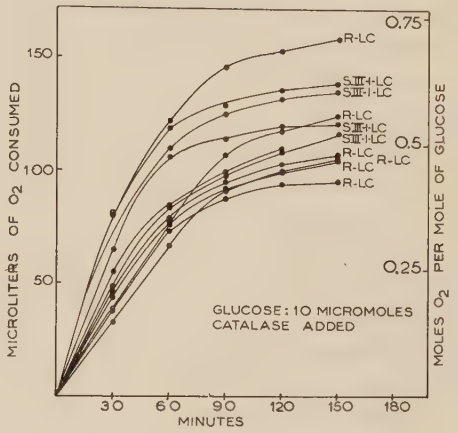


Fig. 8. The respiration of various LC strains of independent origin. Each strain is the product of an independent transformation induced by the LC agent. The variation in the amount of oxygen consumed per molecule of glucose is no greater between these strains than that observed in independent measurements on a single strain.

to oxidize lactate is independent of the capsular constitution of the strain studied, and is strictly correlated with the presence of the LC agent.

3. *Metabolic study of various newly derived LC strains.* With these specific criteria for identifying LC strains at hand, there remained to be established whether LC clones derived from independent transformations, and identified morphologically, constituted a homogeneous physiological class. Therefore, many LC clones were studied manometrically. The clones were obtained from various sorts of transformations: R to R-LC, SIII-1 to SIII-1-LC, and SIII-1 to SIII-N-LC. The inducing agents came from strains A66, 36E and 30A. Strain A66 is the natural source of the LC agent, strain 36E is an SIII-N-LC strain produced by a double transformation of strain SIII-1-T50, and strain 30A is an SIII-N-LC strain produced by single transformation of an SIII-1-LC strain.

In every instance, a clone which had been identified on morphological grounds as LC, proved to have the metabolic characteristics of the LC strains described in the preceding two sections. For example, every LC clone isolated from the experiment shown in Table I was tested manometrically, and proved to have metabolic properties characteristic of LC strains.

Fig. 8 shows a plot of the measurements made on 11 different LC strains of independent origin. The amount of oxygen consumed per molecule of

glucose varied from 0.4 to 0.75, but in no case approached the value of 1.6, characteristic of the non-LC strains. The variation between strains, shown in Fig. 8, is no greater than that observed with a single clone on different days. Many of these strains were also studied with respect to ability to oxidize lactate, and proved not to respire it.

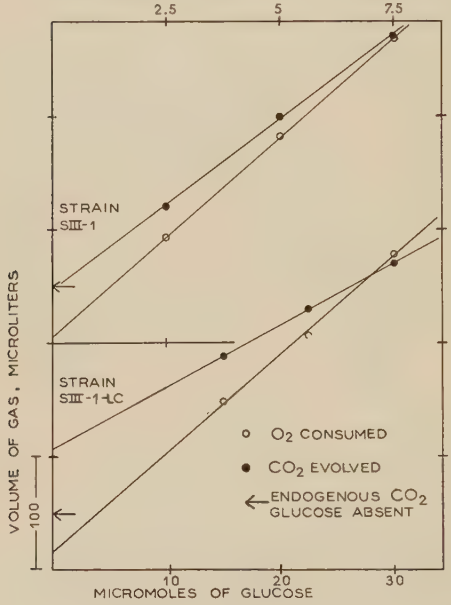


Fig. 9. The determination of respiratory quotients of LC and non-LC strains in the presence of different quantities of glucose. The LC strain has a pronounced endogenous CO_2 production which takes place only when glucose is added. The RQ values for the lower pair of lines are 0.58, 0.62, and 0.61; for the upper pair of lines, 0.74, 0.82, and 0.82.

The experiments demonstrate, therefore, that those clones which have been called LC because of the characteristics of their colonies constitute a distinct and homogeneous group with respect to their metabolic properties. Since the presence or absence of a capsule in no way influences these properties, we can say that the LC agent exhibits a complete physiological autonomy with respect to the capsular transforming agent.

4. *Respiratory quotients of strain A66, and LC and non-LC strains.* Respiratory quotients were determined on various of the strains described above, in order to further characterize them. Strains A66 and SIII-1-T50, when studied by the direct method, gave very reproducible values, 0.95 and 0.8 respectively. However, an LC strain derived from SIII-1-T50 proved exceedingly variable, until it was found that endogenous CO_2 production was not the same in the presence and in the absence of glucose. The technique was then adopted of running three different concentrations of glucose simultaneously, and extrapolating for both endogenous respiration and CO_2 production.

Values ranging from 0.5 to 0.6 were then obtained. Fig. 9 shows the results of such an experiment.

The RQ of strain A66 is typical for lactic bacteria. The RQs of strain SIII-1-T50 and the LC strain derived from it are not. A more striking deviation from the classical picture of respiration is the high endogenous CO_2 production by the LC strain, which takes place only in the presence of glucose.

5. *The formation of lactic acid from glucose by an LC strain.* It was pointed out to me by Dr. H. Kalckar that since LC strains do not oxidize lactate, they might also fail to form lactic acid by fermentation of glucose. An experiment was therefore devised to test this point. An LC strain, derived from transformation of strain SIII-1-T50, was seeded simultaneously into two 300 cc erlenmeyer flasks, the one containing 200 cc of basal medium (medium 1, reference 4), and the other containing the same medium to which a measured amount of glucose was added. These cultures were incubated for two hours after appearance of turbidity, and placed in the refrigerator until the next day. The bacteria were then removed by centrifugation (1 hour), and the supernatant media decanted carefully. A portion of each supernatant liquid was treated with $CuSO_4$ and $Ca(OH)_2$ to remove interfering substance, and the amount of lactic acid determined in each by the method of Friedmann and Kendall. The difference in lactic acid content of the two culture media

TABLE III

Determination of the amount of lactic acid formed by strain SIII-1-LC from a given amount of glucose. The basal medium contained an unknown amount of lactic acid and substances capable of giving rise to lactic acid in the course of bacterial growth. Lactic acid present after growth was compared in basal medium, and in medium to which a known amount of glucose was added at the time of inoculation.

Solution analysed	mg lactate expected	mg lactate found	Per cent recovery
Calcium lactate	1.22	1.18	97
		1.19	97.7
Supernatant liquid, Basal medium	x	2.26	
		2.07	
		2.35	
		Av. 2.23	
Supernatant liquid, Basal Medium plus glucose	x + 1.75[1]	3.485	
		3.63	
		Av. 3.58	77

[1] Assuming that 1 molecule of glucose gives rise to 2 of lactic acid.

is presumed to be equal to the amount of lactic acid formed from the added glucose, in the course of bacterial growth. This procedure was adopted since the basal medium itself is of unknown composition. Table III shows the results of the single experiment performed. The difference in the lactic acid contents of the two media indicates that 77 per cent of the added glucose was recovered as lactic acid at the end of growth. Thus, judging from this one experiment, the conversion of glucose to lactic acid by an LC strain growing in unaerated medium follows the classical picture first described by Hewett (6, 7) for pneumococci and streptococci. The result indicates, consequently, that formation of lactic acid from glucose by glucolyses is essentially normal in the LC strain studied.

DISCUSSION

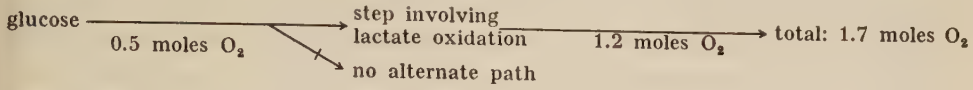
The present study has revealed the presence of a new transforming agent in the desoxyribonucleic acid of the Type III pneumococcal strain A66. This brings to four the number of naturally occurring agents which can be detected in this material: the Type III capsular agent (2), the rough agent (14), the M protein agent (1), and the LC agent. One more element of what must be an exceedingly complex mixture has thus been identified.

The LC agent could be identified owing to two fortunate circumstances. First, the unencapsulated strains usually employed in transformation studies happen to lack the agent, while the Type III strain from which transforming extracts are customarily prepared possesses it. Second, acquistion of the LC agent by non-LC pneumococci is accompanied by a slight, but unmistakable change in colony morphology.

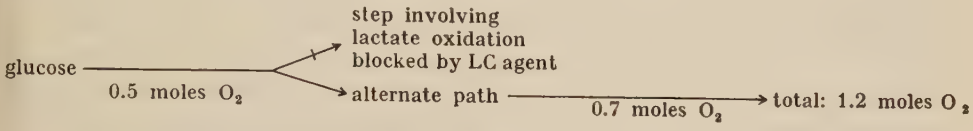
It was possible to show that in addition to altering colony morphology, the LC agent profoundly changes the metabolic pattern of the pneumococci which acquire it through transformation, for such pneumococci lose entirely the capacity to oxidize lactate, and manifest a very reduced respiration of glucose. The LC agent is the first transforming factor whose action has been shown to affect catabolic reactions.

It is not possible from the data presented here to propose a detailed hypothesis to describe the oxidative pathways in the various strains studied. In a general way, however, the results obtained can be drawn together into a coherent scheme on the assumption that the initial steps of glucose oxidation are identical in all strains studied, and that the strains which have been transformed by the LC agent can perform only this initial oxidation step. One then obtains:

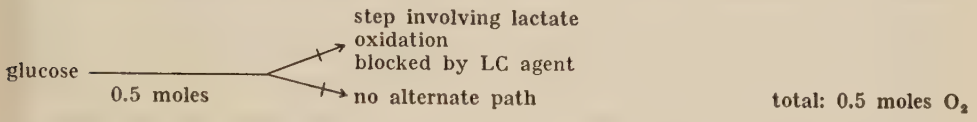
a) Pattern common to strains R36A and SIII-1-T50, and encapsulated strains derived from them by transformation, except LC strains.



b) Pattern in Type III strain A66.



c) Pattern common to strains of type *a* which have acquired the LC agent.



The CO_2 released in the three kinds of oxidations is respectively 1.35, 1.1 and 0.25 molecules per molecule of glucose. Thus, assuming again that the residual respiration of strains of type *c* is the first respiration step, common to all strains, the RQ of the oxidation beyond the first step is 1.1/1.2 for type *a*, and 0.58/0.7 for type *b*. It should be added that the values found for O_2 uptake were variable in strains of type *c*, ranging from 0.3 to 0.7, usually falling between 0.4 and 0.5 molecules per molecule of glucose. This suggests a marked influence of endogenous factors on respiration of these strains. In this connection it should be recalled that in type *c* strains, the addition of glucose stimulates the formation of CO_2 of endogenous origin, indicating very clearly the existence of important endogenous factors.

In all strains studied, the amount of H_2O_2 recovered was found to be less than would have been expected if all hydrogen transfer to oxygen gave rise to hydrogen peroxide. Strains with respiration of type *a* accumulate enough H_2O_2 in the vessel to permit one to say that 70–80 per cent of respiration gives rise to peroxide. Strains with respiration of types *b* and *c* give rise to enough free hydrogen peroxide to account for only 30–40 per cent of the respiration producing peroxide. This is a surprising result if it is true that no catalase or peroxidase activity is present in pneumococci, and that all respiration proceeds *via* flavoprotein enzymes. Recently, evidence has been obtained for a peroxidase-like activity in *Streptococcus faecalis*, as a consequence of which lactate is oxidized by hydrogen peroxide, giving rise to acetate, CO_2 and water. This reaction was shown to be enzymatic (3).

Such a reaction was sought for in the pneumococcal strains studied here, but no evidence could be found for its existence. In the course of these experiments, it was seen that when 16 micromoles of H_2O_2 are added to pneumococcal suspensions, none of it disappears in the course of two hours at 28° C. This suggests that destruction of peroxide by non-enzymatic reactions cannot account for the low recoveries of hydrogen peroxide in the respiration experiments.

A last point to be mentioned is the observation that an LC strain which fails to oxidize lactate does nevertheless produce lactic acid from glucose in the course of growth in non-aerated cultures. This is of some interest, for it has been generally supposed that the metabolism of lactate is mediated by a single enzyme, lactic dehydrogenase, which can either initiate hydrogen transfer from lactate to oxygen, or cause lactate to be synthesized through the reduction of pyruvate (see Ochoa (11), for a review of the various origins of lactate in intermediary metabolism). The characteristics of the LC strains studied here permit one to suppose that lactate arises through the activity of an enzyme which is incapable of mediating its oxidation. Thus, in those strains which do oxidize lactate, two independent enzymes may be involved. On the other hand, one might also suppose that the block introduced by the LC agent affects solely the transfer of hydrogen to oxygen, without affecting the other roles of lactic dehydrogenase, and thus avoid the necessity of postulating a second lactate-activating enzyme.

The finding that lactic acid is formed in normal amounts in the course of growth of an LC strain in glucose-containing medium is of interest for still another reason. One might have supposed that the LC agent blocks glucose metabolism at a very early stage, and that the enzyme (s) for oxidizing lactate fail to be synthetized owing to the absence of lactic acid to serve as an adaptive substrate. However, this cannot be the case since lactate is recovered from the growth medium in amounts typical for homolactic fermenting organism, and presumably is accumulated during growth.

Since both rough and Type III smooth strains have been cultured for many years under essentially identical conditions, it is somewhat surprising to find such a striking difference between them with respect to glucose and lactic acid metabolism. It could, of course, be supposed that the presence or absence of a capsule plays a critical role in determining which kind of metabolism is most advantageous. However, the answer is not so simple. The rough strain R36A was derived originally from an encapsulated Type II strain, D39. Examination of D39 was not undertaken in detail, and a few experiments performed with it have not been included in the experimental

section of the present report. Nevertheless, the data which were obtained showed that strain D39 resembles R36A in that it, too, oxidizes lactate. However, it differs from the rough strain and resembles the Type III strain A66 with respect to the quantity of O_2 consumed per molecule of glucose. Therefore, the loss of a capsule may, at best, have favored the selection of a pneumococcal variant possessing an increased ability to oxidize glucose. One is still faced with explaining how strains cultivated under identical conditions can differ with respect to ability to oxidize lactic acid. At the cellular level, no interference seems to exist between respiratory pattern and the secretion of either Type II or Type III capsule, for it was possible to make by transformation Type II strains which did not oxidize lactate, just as it was possible to make Type III strains which did. The relative selective values of the different combinations of characters were not studied, however, and it is still possible that the type of capsule secreted by a given cell plays an important role in stabilizing in populations a particular kind of respiration pattern. It is more likely, however, that other factors acting in the course of time have brought about the stabilization of different metabolic patterns in the various naturally occurring lines of pneumococci.

SUMMARY

Evidence was obtained which indicates that the normal Type III capsular transforming agent of pneumococcus acts in an all-or-none fashion, at least in the majority of transformations which it induces. No indication could be found to suggest that these inductions produce a spectrum of bacteria having graded abilities to secrete polysaccharide.

In the course of studying the transformations induced by this agent, a new transforming agent was found to exist side by side with the capsular agent in transforming extracts prepared from Type III strain A66. The new agent was detected because the rough strain used in transformation studies lacks this agent.

The new agent is called the LC (large colony) agent because its most obvious manifestation is to increase the size of the colonies formed on petri dishes by pneumococci containing it.

In transformations, the LC agent is usually acquired independently of the capsular agent. Very rarely, both capsular and LC agents are acquired by a single bacterium, in the course of a single treatment of a culture with the transforming extract.

Metabolic studies show that pneumococci which possess the LC agent have

a diminished ability to respire in the presence of glucose, and fail to oxidize lactate. Furthermore, they produce less hydrogen peroxide in the course of glucose oxidation than do pneumococci which do not possess this agent.

The metabolic characteristics of LC and non-LC strains are uninfluenced by the kind of capsule which they possess (Type III, normal or mutant, and Type II).

Applying metabolic criteria to the study of many clones derived independently by transformations with the LC agent and identified on morphological grounds, it is found that these constitute a homogeneous class with respect to the metabolic properties examined.

The LC agent is thus responsible for a particular pattern of metabolism, perhaps produced by the introduction of a block in the oxidation of lactic acid.

A typical LC strain produced by transformation was found to have the following additional characteristics: a) a pronounced formation of CO_2 from endogenous sources which takes place only in the presence of added glucose; b) an RQ atypical of lactic acid bacteria; and c) the ability to form enough lactic acid from glucose in the course of growth in unaerated cultures to acccount for about 77 per cent of the carbon of the added glucose, this in spite of being unable to attack lactate oxidatively under the conditions of the Warburg experiments.

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Experimental Cell Research, **6**, *117–126 (1954)*

THE PERMEABILITY OF NUCLEATED AND ENUCLEATED FRAGMENTS OF *AMOEBA PROTEUS* TO D_2O

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Received May 25, 1953

In a recent review (17) it was concluded that we are still unable to assign a definite function to the nucleus in the cell when it is not dividing. Currently, two experimental approaches are being brought to bear on this problem: the study of the localization of biochemical systems in the isolated nucleus (e.g. 10, 24) and the older approach of comparing the functional capacities of cells with and without nuclei (e.g. 2, 7). The former type of approach may be expected, as techniques improve, to identify the immediate biochemical contribution of the nucleus to the activities of the cell, while the second should permit us to trace the course of events whereby the nucleus determines not only the survival but also the specific character of the cell.

The "morphogenetic" manifestations of the action of the nucleus are at the same time the most readily recognized and most complex evidences of nuclear function. To some extent at least they represent *continuous* activity of the nucleus on the cell, for experiments on nuclear transplantation (4, 9, 14) consistently show that a "foreign" nucleus will impose some of the specific characteristics of the type from which it is derived on an already-differentiated cell of another type. The fact that the action of the nucleus is reciprocally affected by the cytoplasm in which it finds itself, as was shown so elegantly in recent experiments on *Paramecium* (19), concerns us less in the present discussion than the fact that the activity of the nucleus must be viewed as continuous. A continuous "morphogenetic" action of the nucleus implies that it is ultimately implicated in the maintenance of the organization of the cell at the level of structure as well as at the simpler biochemical level described in terms of enzyme and coenzyme formation, etc.

Granted that the problem of the relation between the nucleus and cell structure is a legitimate one, the next consideration is that of experimental design. The logical experimental design is to measure the effects of removing the nucleus on the finer structure somewhere in the cell. The difficulty is that when we formulate a problem involving structure in terms of differences between the normal and the abnormal, we realize how completely inadequate

[1] Supported by grants from the American Cancer Society, recommended by the Committee on Growth, National Research Council, and from University of California Cancer Research funds.

our present knowledge of structure is. In most cases we could not do the proposed experiment because we would not know what to look for.

In the present instance we have turned to the cell surface as the most accessible structured region of the cell, the one concerning which we have the most information and the best methods of measurement. Optical data, electron microscopic data, electrical data, and the body of knowledge concerning permeability may be brought together into models of the molecular organization of the cell surface that have at least internal consistency (e.g. 8, 21). The so-called "passive permeability", which does not seem to involve the performance of osmotic work, presumably reflects the molecular organization of the surface and might be used as a detector of effects of the nucleus on the surface as it has been used as a detector of other physiological variables.

A few studies have been made on the consequences of enucleation for cell permeability. It has been shown that separation of the nucleus does not affect the permeability of *Arbacia* eggs to water (16), or the permeability of *Spirogyra* cells to glycerol (10). These have been short-term experiments but, as far as they go, show that the nucleus is not *immediately* implicated in permeability phenomena.

In the present case, the permeability of nucleated and enucleated halves of *Amoeba proteus* to heavy water (D_2O) was measured for a period of up to 38 hours following the cutting of the cells, and compared with that of intact cells. *Amoeba proteus* has been used extensively in recent years for the study of the consequences of enucleation, and it is known that by 24 hours after enucleation there are defects in phosphorus metabolism (18), a lowered RNA content (13), and an abnormally high amylase activity (27). On the other hand, the activity of some enzymes remains unaffected (26). In the related *Amoeba sphaeronucleata*, it is known (4) that the cells may be restored to their normal condition if a fresh nucleus is grafted into the enucleated cells within three days, so that it seems unlikely that we are dealing with cells in which the effects of the absence of the nucleus have reached the point of irreversibility.

It seems, therefore, safe to assume that in a half-amoeba that has been without a nucleus for 24–38 hours we are dealing with a cell in which the detrimental effects of the absence of a nucleus have asserted themselves, but which has not gone so far in its decline that we are dealing with mere manifestations of moribundity. The question is whether abnormalities at the structural level may be found by studying the behavior of the surface as a barrier to water.

METHODS

The heavy water indicator technique was combined with the diver balance in a manner described by Pigón and Zeuthen (22) and Løvtrup and Pigón (15) to determine the rate of movement of water across the surface of whole cells and nucleate and enucleate halves of *Amoeba proteus*. The method offers several advantages over osmotic methods of determining water permeability: 1) It does not involve measuring a change in volume of the cell, which is difficult to do accurately except on cells of very regular geometric shape. 2) No stretching, shrinking, or formation of new membrane is involved. 3) It is not necessary to know the osmotic pressure of the cell cytoplasm. 4) With cells which show significant salt leakage (ovarian eggs, marine eggs, etc.), the determination of water permeability can be carried out in a medium isotonic with the cell cytoplasm.

Any difference between the permeability constants of D_2O and H_2O would be of no consequence in a comparative study such as the one we are reporting. The possibility of injurious effects of D_2O does not seem to be serious in this case. We have kept *Amoeba proteus* in 20 per cent D_2O for as long as 96 hours, and have observed that they divide in this medium.

A. Determination of the permeability constant. The change in the reduced weight of a single amoeba or one or two halves introduced in Brandwein's medium (3) containing 15 per cent heavy water was followed by means of the Cartesian diver balance. The actual experimental values are in terms of the equilibrium pressure difference between the empty diver balance and the diver loaded with an amoeba, and are converted into values of reduced weight by equation 1 in (28). A hollow glass bead was used for the standard.

The permeability constant for heavy water was calculated from an equation based on Fick's Law for diffusion as described in some detail by Løvtrup and Pigón (15) and slightly simplified by Prescott and Zeuthen (23):

$$\log \Delta RW = \frac{-P\,Q}{2.3026}\,t + \log C\,(d_{D_2O} - d_{H_2O})\,V_{H_2O} \qquad 1)$$

ΔRW is the change in reduced weight of the amoeba after t seconds in the heavy water medium, P is the permeability constant (μ-sec^{-1}), Q is the surface area (μ^2) to volume (μ^3) ratio of the amoeba, C is the concentration of D_2O in the experimental medium expressed as a per cent, V_{H_2O} is the water volume of the amoeba (μ^3), and $(d_{D_2O} - d_{H_2O})$ is the density difference between pure heavy water and ordinary water. The volume and surface

area were determined by microscopic measurements, using amoebae that had become approximately spherical in form as a result of being gently agitated.

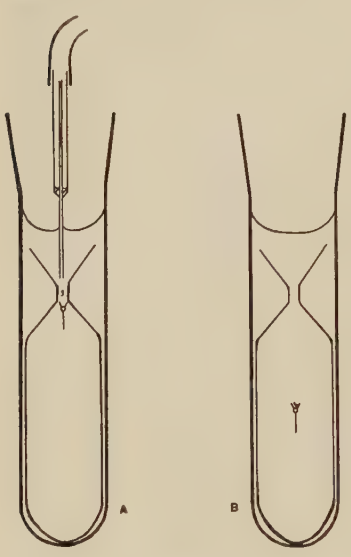

Fig. 1 (a) Loading the diver with an amoeba which has just been released from a braking pipette and is falling through the constriction in the flotation vessel to the diver below. (b) The loaded diver in position for equilibrium pressure readings. Diver and amoeba not drawn to scale.

The total amount of exchanging water in the amoeba can be calculated from the equation:

$$V_{H_2O} = \frac{\Delta RW_\infty}{d_1 - d_2} \qquad 2)$$

in which V_{H_2O} is the volume of exchanging water, ΔRW_∞ is the change in RW of the amoeba after infinite time in the heavy water medium, and $d_1 - d_2$ is the density difference between amoeba medium containing 15 per cent D_2O and plain amoeba medium. The assumption is made that the amount of water in the amoeba taking part in the heavy water exchange is very nearly equal to the absolute amount present. As will be seen below, this assumption seems justified.

B. The diver balance. The diver balance was constructed essentially in the manner described by Zeuthen (28). An extremely thin-walled pyrex capillary tube with an outside diameter of about 40 μ was sealed off with a hot nichrome wire, with great care to avoid the slightest accumulation of glass at the seal. The sealed tip of the capillary was brought into a microflame and the bulb of the diver blown quickly in the flame and withdrawn. Divers small enough to measure accurately the RW of a single *A. proteus* are very difficult to

make, but if handled with care they can be used over and over with occasional cleaning in 95 per cent ethyl alcohol or concentrated sulphuric acid.

The operation of loading and unloading the diver balance with an amoeba was greatly simplified by the use of a flotation vessel constricted near the top so as to form a narrow neck with a bore diameter just greater than the diameter of the cup of the diver balance. For loading, the diver was brought up to the lower end of the constriction in the flotation vessel and the amoeba was dropped from a braking pipette just above the constriction. The operation is illustrated in Fig. 1 a in which an amoeba is shown dropping through the constriction to the diver balance below. The short fall also serves to wash the amoeba free of any pure ordinary water introduced with it. Fig. 1 b shows the loaded diver in the position for equilibrium pressure readings. Unloading was accomplished by bringing the diver up into the constriction and gently removing the amoeba with a braking pipette.

MATERIALS

Permeability studies were carried out on whole *A. proteus* and nucleate and enucleate halves at various times after cutting. Whole amoebae were starved for 24 hours prior to measurements of permeability in order to minimize contractile vacuole activity which could complicate the measurements of *RW*.

EXPERIMENTAL RESULTS

A single whole or half amoeba or two halves were dropped onto a diver balance in a heavy water medium, and the *RW* was usually recorded at one minute intervals during the following fourteen minutes. Because of manipulations there was always a one minute delay from the time the amoeba was introduced into the heavy water medium until the first equilibrium pressure recording could be made. However, subsequent recordings of equilibrium pressure were sufficient to establish the curve representing the ΔRW of an amoeba as its heavy water content increased. For convenience in extrapolating the experimentally established curve, the log of ΔRW occurring over succeeding one minute intervals was plotted against the interval (see Fig. 2). The ΔRW was determined from the extrapolated curve. Since more than 99 per cent of the exchangeable water of the amoeba has exchanged with the outside medium by the end of fourteen minutes, ΔRW_∞ is for all practical purposes equal to the ΔRW occurring over the first fourteen minutes.

The volume of exchanging water of the amoeba varied from 81 per cent to 92 per cent of the total amoeba volume in the seventeen experiments carried

out. There was no difference in the relative water volume of whole or half amoebae. It is evident that the heavy water exchange involves nearly all of the water within the amoeba. From the character of the exchange curve it is also reasonable to conclude that heavy water, once within the plasma membrane, diffuses freely throughout the cytoplasm. The six values for the heavy water permeability constant for whole amoebae, the three for nucleate halves, and the eight for enucleate halves are all included in Table I. The latter values are for enucleate halves at various times up to 38 hours after cutting. Typical exchange curves for a whole amoeba (# 11 in Table I) and for two enucleate halves (# 16 and # 17 in Table I; weighed together as one experiment) are represented in Figs. 2 and 3.

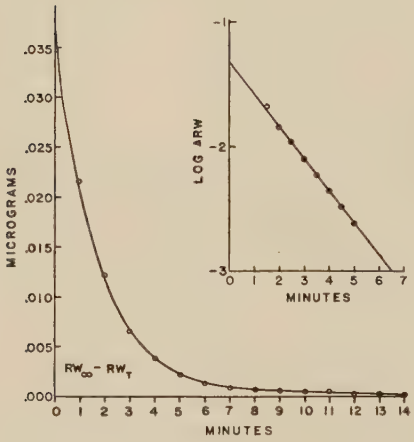

Fig. 2.

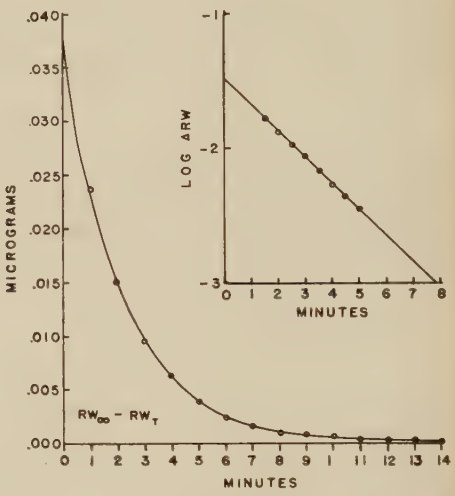

Fig. 3.

Fig. 2 (left). Time course of *RW* and log *RW* of a whole amoeba following immersion in medium containing 15 per cent D_2O. (Amoeba #11 of Table I.)
Fig. 3 (right). Time course of *RW* and log *RW* of two enucleated half amoebae following immersion in medium containing 15 per cent D_2O. (Enucleated halves #16 and #17 of Table I.)

Usually the permeability constant was determined from rates of penetration of D_2O into amoebae. Two experiments were carried out in which a whole amoeba was first charged with 15 per cent heavy water, and the rate of loss of D_2O from the amoeba placed in a non-heavy water medium was determined on a diver balance. These experiments are represented by amoebae # 31 and # 32 in Table I. The constants thus determined indicate that the rate of diffusion of heavy water across the plasma membrane is the same in both directions.

In calculating P from equation 1) it was convenient to substitute for t the time at which half of the exchangeable amoeba water volume had exchanged with the outside medium. ΔRW in equation 1) then becomes one half of ΔRW_∞. It is surprising that in spite of the retardation of diffusion caused by plasma membrane, one half of the water volume of an *A. proteus* exchanges with water of the outside medium every ninety seconds.

TABLE I

Heavy water permeability constants of whole *Amoeba proteus*, nucleated halves, and enucleated halves. Numbers designate individual amoebae or fragments. Where the same number appears in all three columns, they refer to experiments in which the permeability constant was measured before cutting, and measured later on the fragments of the same cell after cutting. Two numbers appear when measurements were made on two fragments.

Whole amoebae	P (μ/sec)	Nucleate halves	P (μ/sec)	Enucleate halves	Hours after cutting	P (μ/sec)
#6	0.17	#10	0.28	#11	½	0.16
#8	0.20	#11	0.17	#11	24½	0.16
#9	0.23	#12 + #13	0.20	#12 + #13	¼	0.26
#11	0.22			#12 + #13	27	0.21
#31	0.24			#16 + #17	32	0.23
#32	0.19			#20 + #21	27	0.21
				#26 + #27	24	0.24
				#28 + #29	38	0.24
Av.	0.21	Av.	0.22	Av.		0.21

Orr and Butler (20) and Temkin (25) have reported a value for the free diffusion constant of D_2O in H_2O which has been interpolated for 20°C by Løvtrup and Pigón (15) to 2.2×10^3 μ² sec^{-1}. If we assume that the plasma membrane of *A. proteus* has a thickness of 0.1 μ, then free diffusion of D_2O has been retarded by the membrane by a factor of 10^5. As pointed out by Løvtrup and Pigón (15) it is impossible to say whether $1/10^5$ of the surface of the amoeba is available for free diffusion through a watery phase or whether diffusion occurs across the whole extent of the plasma membrane at a rate reduced by a factor of 10^5.

DISCUSSION

These measurements, showing a surprising degree of consistency in view of the fact that they represent individual free-living cells rather than statistically homogenized populations, show no evidence that the permeability of the cell surface to water is affected when the cell has been deprived of its

nucleus for up to 38 hours. By precedent, this is not unexpected, in view of past failures to detect physiological lesions in enucleated cells. But in this case, some of the history of the cell following enucleation is known and the method is such that moderately small quantitative differences might have been detected.

The unchanged permeability to water suggests a high degree of chemical stability or else a high degree of independence from those processes that *are* being affected by the absence of the nucleus during the first 38 hours. For instance, the total cytoplasmic RNA is down by 10–20 per cent (13, confirmed in this laboratory by T. W. James). Recently, it has been suggested by Lansing and Rosenthal (12) that RNA is a constituent of the cell surface and is involved in ion transport. On the basis of our findings, it must be concluded either that the RNA molecules of the surface are metabolically more stable than the average RNA molecule of the cytoplasm as a whole, or that surface RNA is not involved in the structural attributes of the surface that govern the movement of water.

Likewise, it is now well established that, in amoeba, the incorporation of phosphorus into the organic constituents of the cell declines rapidly after enucleation. Mazia and Hirshfield (18) showed that the uptake of P^{32} in the first 24 hours by enucleated halves of *A. proteus* was one third that of the nucleated halves. These observations have been confirmed by Brachet and collaborators (2). Evidently, a decrease in total phosphorus turnover has no impact on the structure of the cell surface in so far as water penetration reflects this structure.

More recently, Mazia and Bendix (unpublished) have made a study of the effect of enucleation on the incorporation of phosphorus into lipids. They find that the effect is quantitatively the same as for total phosphorus: the incorporation of P into lipid declines to about one third the level observed in nucleated cells. Therefore, we must conclude that the phospholipids play no part in the cell's barriers to water penetration or that the small fraction of the phospholipid that is involved in the structure of the surface is metabolically more stable than the rest. Most pictures of the molecular organization of the plasma membrane assign to the lipids (and the phospholipids predominate quantitatively in the surface) a decisive role in the barrier to water, whether it be a continuous lipid layer (8) or a "pore" in a lipoprotein layer (21).

The more superficial physiological lesions consequent to enucleation deserve comment. All observers have noted the sluggish locomotion, abnormal pseudopod formation, feeble capacity for food capture of the enucleated amoeba. These defects become very evident within several hours after

enucleation. It might be thought that they involve differences between the surface of the nucleated and enucleated cell, but if this is the case, the differences apparently are not such as would influence water permeability. Recently, Goldacre (6) has proposed an interesting theory of amoeboid movement according to which the proteins of the surface would be continuously replenished at the forward end of the cell by proteins from the cytoplasm while at the posterior end the surface proteins would be returned to the cytoplasm. This surface turnover would be less in slowly-moving or immobile cells. One of us (D.P.) has confirmed the observations on amoeba on which Goldacre's hypotheses are based. We would have to conclude from the present study that the postulated turnover of protein at the surface of amoeba does not seem to be related to a surface barrier to water.

It is hardly profitable to pursue the consequences of a negative result farther than, if as far as, the above discussion. The essence of the situation is that the barrier to water which exists at the cell surface seems to be remarkably stable in the metabolic sense. From the standpoint of the "morphogenetic" action of the nucleus, we must conclude that this aspect of the structural organization of the cell is either independent of nuclear influence, or that the nuclear influence will express itself slowly and at a rate determined by whatever small capacity for "turnover" that the barrier to water possesses.

Davson and Danielli (8; p. 117) have compared the water-permeability constants of various types of cells as obtained by osmotic methods. Prescott and Zeuthen (23) have made a similar comparison of data obtained by measurements of heavy water diffusion. The differences between the constants obtained by the two methods do not concern us here, but the comparisons of cells of varying origin do. While species differences do exist, the orders of magnitude are remarkably uniform among animal cells (with the well-known exception of the mammalian erythrocyte). Variations in water permeability are apparently greater among plant cells (1). It has been found (22) that the D_2O permeability of the giant amoeba *Chaos chaos* is very close to that which we have determined on *Amoeba proteus*. We have not yet had the opportunity to measure the permeability of other species of amoebae. The tendency toward uniformity among animal cells of varied origin might suggest that the molecular organization of that part of the cell surface that is concerned with the penetration of water is not, after all, an expression of a specific "morphogenetic" control, but is a simpler consequence of chemical conditions in the cytoplasm that are common to many types of cells. It appears that an adequate picture of the cell membrane will have to meet the requirement that it permits the permeability to water to be more

uniform than the permeability to solutes. It is not clear to us whether this has been considered in the formulation of membrane structure to which we have referred above. In the extension of the present experiments, the need for studying the impact of enucleation on the permeability to passively penetrating solutes on the one hand and actively transported ions, etc. on the other, becomes obvious.

SUMMARY

The Cartesian Diver Balance method of Pigón and Zeuthen was adapted to the measurement of the D_2O permeability of single *Amoeba proteus* and of nucleated and enucleated fragments. The permeability constants of half cells that had been without a nucleus for 38 hours remained identical with those of nucleated half cells and of intact amoebae.

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Experimental Cell Research, **6**, *127–133 (1954)*

CHEMICAL AND HISTOCHEMICAL DEMONSTRATION OF ACID ESTERS BY ACETIC IRON REAGENT

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Received June 1, 1953

Acetic iron reagent was used by Hale (7) for the detection of hyaluronic acid and other acid mucopolysaccharides. On the other hand, Lillie and Mowry (11) have shown that gastric mucin, reticulin and collagen in sections of rabbit tissues were also selectively impregnated by iron. This may, however, be due to the presence of acid polysaccharides. However, Pearse (13) and Davies (4) have found this method to be nonspecific, because the Prussian blue reaction was positive with many other substances such as casein, egg albumin, fibrin, peptone, etc.

The author wished to ascertain whether the acetic iron reacts generally with sulphuric and phosphoric acid groups when present as acid esters, and thus whether this reagent would react readily with acid polysaccharides, polynucleotides, phosphoproteins and phospholipoids.

RESULTS

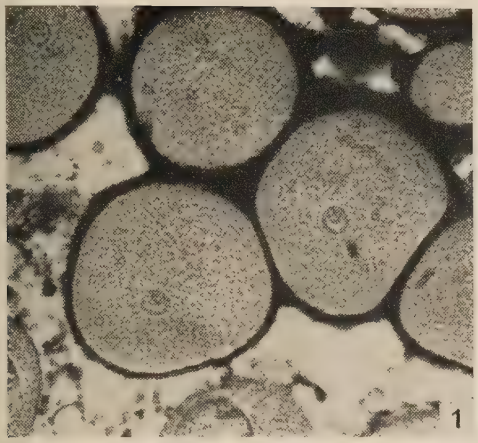
Chemical experiments. The contents of sulphate (14, 16) and phosphorus (1) were determined in the substances used in the experiments. For the determination of sulphate the substance was hydrolyzed with 0.2 *N* hydrochloric acid in an autoclave for 1 hour at 2.5 atmospheres (126°C). The hydrolysate was neutralized with sodium hydroxide. The content of iron was tested in the specimen both before and after addition of acetic iron reagent. In the former case all substances were free from iron but in the latter they gave maximal reaction. Many substances, for example phospholipoids and polyuronides, were purified by fractionated precipitation (lecithin, alginic and pectic acids) and recrystallization (sphingomyelin). Pectic acid was prepared from pectin of *Citrus*. Pectin was dissolved in water, dialyzed against distilled water for about 48 hours, then the insoluble part of pectin was centrifuged off and the viscous solution was diluted with acidified acetone or ethanol until the precipitate was formed. The precipitate was isolated, washed with alcohol, acetone and ether, and dried. This material was referred to in Table I as the pectic substance. Further, the pectic substance was demethylated with 0.1 *N* sodium hydroxide. After demethylation the pectic acid was precipitated by slight excess of hydrochloric acid. The precipitate was washed with acetone, ether and

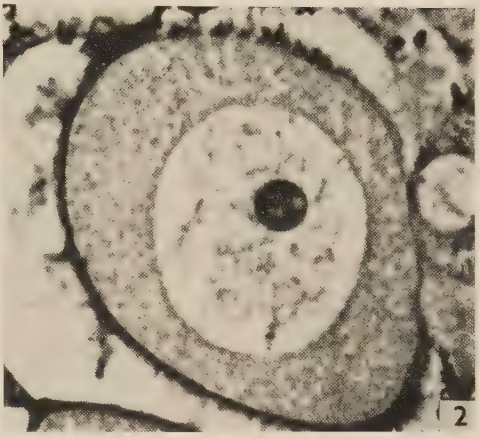
dried. This substance was referred as pectic acid. Hyaluronic acid was purified by means of electrophoresis (5).

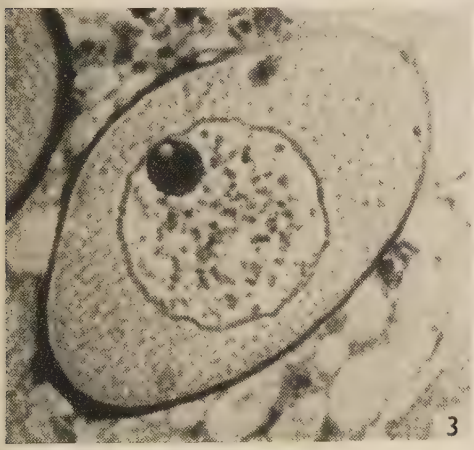
For the reaction with acetic iron 1 per cent solutions or suspensions of the substances in *M* acetic acid were prepared. A small amount of the acetic iron solution (equal volumes of 2 *M* acetic acid and dialyzed ferric hydroxide) was added. The mixture was dialyzed in a cellophane tube against *M* acetic acid until the dialysate was free of iron ions (it could not be dialyzed against water because of the formation of insoluble ferric hydroxide). After this a solution of potassium ferrocyanide was added to the dialyzed solution or suspension to test for the presence of combined iron. That it is possible to detect the acid polysaccharides by acetic iron in chemical experiments is confirmed by the data in Table I.

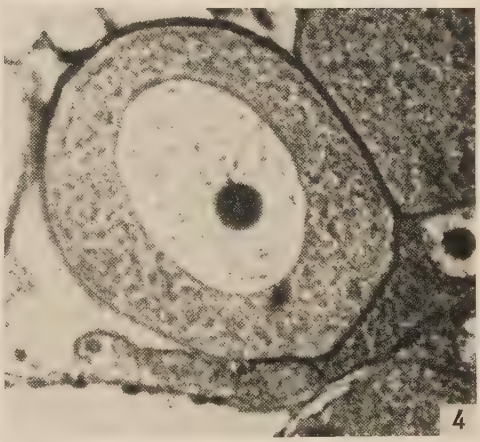
Acetic iron reacts with a sulphate group in organic compounds such as heparin, sea urchin jelly substances (*Strongylocentrotus droebachiensis, Paracentrotus lividus*), and with a carboxyl group of uronic acid residue in alginic, hyaluronic and pectic acids. If the carboxyl groups of a polyuronide are esterified with methyl radicals, e.g., in pectic substance, the reaction with acetic iron is not obtained. However, the same amount of Prussian blue colour is given by ribonucleic acid (RNA), desoxyribonucleic acid (DNA), ribonucleotides of the sea urchin *Echinocardium cordatum* egg, phospholipoids, i.e., substances containing phosphate groups. Casein, which contains only 0.8 per cent phosphorus and where the phosphate is linked as phosphoserine (12) and phosphothreonine (18), produces a strong Prussian blue colour. Likewise, fibrinogen with a phosphorus content of 0.1 per cent gives a strong Prussian blue reaction. It seems that the content of phosphorus is too insignificant in the two specimens mentioned to give so strong a blue dye, although each phosphate group probably reacts with two iron atoms. However, both above-mentioned proteins contain in their molecule free carboxyl radicals of monoaminodicarboxylic acids (casein—glutamic, fibrinogen—aspartic and glutamic acids). These react with acetic iron in a similar way as the carboxyl groups of polyuronides.

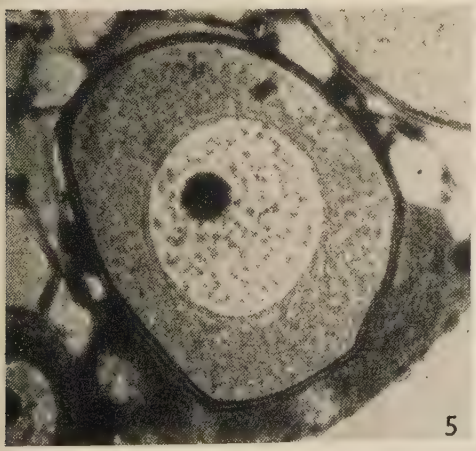
Histochemical experiments. For the histochemical investigation ovaries of the sea urchin *Psammechinus miliaris*, fixed in the Carnoy fluid, and smears of RNA and DNA on microscope slides, were used. With the suspensions of the RNA and DNA in distilled water distinct patterns were obtained on the slide. The water was evaporated and the smears dried at 85°C, or dehydrated with absolute alcohol or acetone. The preparations were stained as described by Hale (7, cf. 6). The staining technique of Hale was modified so that after immersion in the acetic iron solution the sections or smears were washed with *M* acetic acid in order to remove free (nonreacted) iron which would give a diffuse staining if the slides were washed with water. In all smears of RNA and DNA a positive reaction was obtained with acetic iron. In order to compare the staining of the tissues, obtained by Hale's modified technique, with other











Microphotographs of *Psammechinus miliaris* ovaries. Thickness of sections 6 μ.

Fig. 1. Mature eggs surrounded by jelly coat. Magn. 250 ×, Hale + Eosine, red filter.
Figs. 2 to 5. Oocytes. Magn. 500 ×: 2. Ritter and Oleson, red filter, 3. Hale + Eosine, red filter, 4. Methylgreen-Pyronin, green filter. 5. May-Grünwald-Giemsa, green filter.

histochemical stainings and to determine closer the identity of stained substances, the following histochemical methods were used: for RNA and DNA—methylgreen-pyronin (2, 9, 10) and May-Grünwald-Giemsa (9), and for polysaccharides—the Hotchkiss (8) and Ritter and Oleson (15) methods.

Jelly coat. The jelly coat which surrounds the eggs (Fig. 1) or the oocytes (Figs. 2 and 3) and which contains an acid polysaccharide (17), reacts readily with acetic iron reagent and is visualized on the sections by its dark blue staining. By means of the Hotchkiss method a bright red stain was obtained. The sections of the ovaries were oxidized by periodic acid both before and after immersion in the acetic iron reagent, but no difference between the stains produced by Hale's method could be observed. Using the Ritter and Oleson (15) technique the jelly coat was stained violet-blue (Fig. 2).

Nucleolus (Figs. 2 to 5). The nucleolus of the oocyte stained red with methylgreen-pyronin (Fig. 4) and dark blue by the method of May-Grünwald-Giemsa (Fig. 5). This confirms that the stained substance in the nucleolus is ribonucleoprotein (RNP). The nucleolus of the oocyte was also shown to stain strongly blue (see Figs. 2 and 3) with the acetic iron - potassium ferrocyanide. This staining reaction indicates the presence of acid phosphate groups in the nucleolus.

DISCUSSION

The results obtained on the staining ability of the jelly coat of *Ps. miliaris* eggs confirm the findings by Vasseur (17) concerning the structure of the jelly substance. Although his work is carried out on another species, *Echinocardium cordatum*, it seems probable that the structure of these jelly substances is similar, i.e., that they contain a branched polysaccharide component containing one sulphate group for each monosaccharide residue. These sulphate groups in the monosaccharide residues of the acid polysaccharide component in the jelly substance are detected by the Hale histochemical technique as a Prussian blue colour. The jelly coat was also stained by the Hotchkiss method. This staining is caused by the aldehyde groups, which are produced by the periodic acid oxidation of each monosaccharide molecule in the side chains (17). These histochemical reactions thus confirm that the jelly coat contains an acid polysaccharide.

With Hale's acetic iron - potassium ferrocyanide technique the nucleolus of the *Ps. miliaris* oocytes is stained dark blue. According to the chemical

TABLE I

Acetic iron reaction with compounds containing acid groups. The acetic iron solution was added to a solution or suspension of the specimen, the mixture was dialyzed against *M* acetic acid, and 0.5 ml of the dialyzed solution or suspension was added to 0.5 ml 0.02 *M* potassium ferrocyanide in 0.14 *N* hydrochloric acid.

Specimen[1]	Per cent of phosphorus	Per cent of SO_4''	Degree of reaction
Acetic iron solution			–
Compounds containing sulphate groups:			
Heparin—Vitrum	0.4	32.1	+ + +
Jelly coat of *P. lividus*	0.3	21.0	+ +
Jelly coat of *Str. droebachiensis*	0.4	26.5	+ +
Compounds containing phosphate groups:			
Calcium glucose diphosphate—Glucofos	10.0		+ + +
Ribonucleotides of *E. cordatum* egg	7.3	nil	+ + +
DNA of sperm	9.8	,,	+ + +
RNA of yeast	9.2		+ + +
Lecithin—ex ovo	3.8	,,	+ + +[2]
Lysolecithin	5.1	,,	+ + +
Sphingomyelin	0.9	2.9	+ + +
Casein—vitamin-free	0.8	0.1	+ +
Compounds containing uronic acid residues:			
Alginic acid	nil	nil	+ + +
Hyaluronic acid	,,	,,	+ +
Pectic acid	,,	,,	+ +
Pectic substance of *Citrus*	,,	,,	±
Other substances:			
Fibrinogen—Bovine	0.1	nil	+ + +
Potatostarch	0.1	,,	±
Gelatin	nil	,,	±
Glycogen	,,	,,	–
Albumin—cryst. bovinplasma	,,	,,	–

investigation in the present paper the blue colour may be due to the phosphate groups of RNP, RNA, DNA, etc. (Table I). It seems that the phosphate groups of RNP are those which give the blue staining of the nucleolus (Figs. 2, 3), because the methylgreen-pyronin and May-Grünwald-Giemsa methods both gave positive results on the RNP and RNA in the nucleolus (Figs. 4, 5). It

[1] Jelly coat of sea urchin *Str. droebachiensis*—0.1, *P. lividus*—0.07, and other substances 1 per cent solution.

[2] Colour was greenish-blue.

was experimentally established that the nucleolus of the oocyte did not contain acid polysaccharides, at least not the type present in the jelly substance. After treating the histological slides with distilled water for 12 hours at 55°C, or with *M* sodium chloride solution for 24 hours at 37°C, or with *N* hydrochloric acid for 2 hours at 60°C, the jelly coat was completely removed but the nucleolus of the oocyte was stained blue as before when Hale's method was used. The nucleolus of the oocyte, treated by Hotchkiss's technique, did not give a positive test with Feulgen's reagent.

From this work it can be seen that the use of the acetic iron - potassium ferrocyanide reaction for the histochemical investigation as a staining method detects substances containing the acid ester groups of sulphate—$R.O.SO_3^-$, phosphate—$R.O.PO_3^=$, $(R.O)_2 : PO_2^-$, and also the free carboxyl groups, $R.CO_2^-$, of uronic acids and monoaminodicarboxylic acids (as aspartic, glutamic and hydroxyglutamic acids) of proteins.

Hale's method is nonspecific for hyaluronic acid and for mucopolysaccharides esterified with sulphuric acid. The iron acetate reacts in acid solution as readily with other acid polysaccharides, with polynucleotides, with phosphoproteins and with phospholipoids (cephalin, lecithin, sphingomyelin). It may be that with further development of the method, e.g. by combination with fractionated extraction or enzymatical digestion and by using the technique of selective blocking agents (3), it will be found useful for the detection of the different groups of substances mentioned above. Particularly it may be used in combination with other methods and under these conditions give valuable information.

SUMMARY

Hale's method using the Prussian blue colour is based on the specific reaction of ferric iron with acid esters (sulphuric or phosphoric acid) and also with carboxyl groups of uronic acids and monoaminodicarboxylic acid residues of proteins in a slightly acid environment. Positive reaction is obtained with polyuronides, polysaccharide sulphuric acid esters, polynucleotides, phosphoproteins and phospholipoids. The further development of Hale's method is discussed briefly.

The author wishes to thank Professor John Runnström for his interest in this investigation. He is indebted to Dr. Erik Vasseur for his valuable discussion and many generously provided substances for this research. He is also indebted to Dr. Sven Gardell for providing the pure hyaluronic acid and to Dr. Georg Kriszat for help with the microphotographs.

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NUCLEAR STUDIES ON THE SMALLER *MYXOPHYCEAE*[1, 2]

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Received June 11, 1953

The centroplasm of the *Myxophyceae* was first described by Schmitz (46) who considered it to be a nucleus, although later retracting this opinion (47, 48). Numerous subsequent studies indicate that the *Myxophyceae* possess nuclear material, and various bodies have been described within the centroplasm. Fritsch (11) listed the majority of these reports; among

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Figs. 1 and 2. *Synechococcus* fixed with alcohol, treated with N $HClO_4$ at 4°C for 24 hours, stained.
Figs. 3 and 4. Cells from the same preparation as in Figs. 1 and 2, but treated with N $HClO_4$ at 70°C for 20 minutes following the cold $HClO_4$ treatment and before staining.
Figs. 5 to 9. Stained preparations of *Oscillatoria* showing nuclear material.
Figs. 10 to 20. Nuclear organization of *Chroococcus* in stained preparations.
Figs. 21 to 28. Stained cells of *Synechococcus*. Rodlike chromatinic elements are particularly apparent in Figs. 27 and 28.
Figs. 29 to 32. Stained filaments of *Fremyella* showing nuclear material.
Figs. 33 to 36. Stained cells of *Nostoc*. The unusual appearing cells in Figs. 35 and 36 are heterocysts. The rod-shaped element associated with the heterocyst in Fig. 36 is a piece of superimposed, extraneous material.
Figs. 37 to 41. Stained cells of *Phormidium* showing various nuclear arrangements. Note the beaded strings of chromatinic material in the lower cells of Fig. 39.
Figs. 42 to 49. Stained cells of *Microcoleus* showing nuclear arrangements. Figs. 47 to 49 are single cells detached from a filament, and viewed through the longitudinal axis of the filament. The two nucleoluslike spots in Fig. 43 result from air bubbles on the photographic negative.
Figs. 50 to 53. Stained cells of *Gloeocapsa* showing nuclear material.
Figs. 54 to 57. Stained cells of *Anabaena* showing nuclear elements. The bridged cells in Figs. 55 and 56 are heterocysts.
Figs. 58 to 65. Living cells of *Chroococcus* showing the nuclear regions. Figs. 63 to 65 show successive stages in cell division.
Figs. 66 to 68. Living cells of *Nostoc* showing the nuclear regions. A heterocyst is shown in Fig. 66.
Figs. 69 to 77. Living cells of *Synechococcus* showing the nuclear regions. Note the rodlike elements in Fig. 76.
Figs. 78 to 82. Living cells of *Microcoleus* showing the nuclear regions. Detached cells can be seen in Figs. 80 and 82.
Figs. 83 to 86. Living cells of *Fremyella* showing the nuclear regions.

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[1] This investigation was supported by a fellowship granted by the Philadelphia Lager Beer Brewers' Association.

[2] A preliminary report of this investigation appeared in *Proc. Natl. Acad. Sci.*, **38**, 61, 1952.

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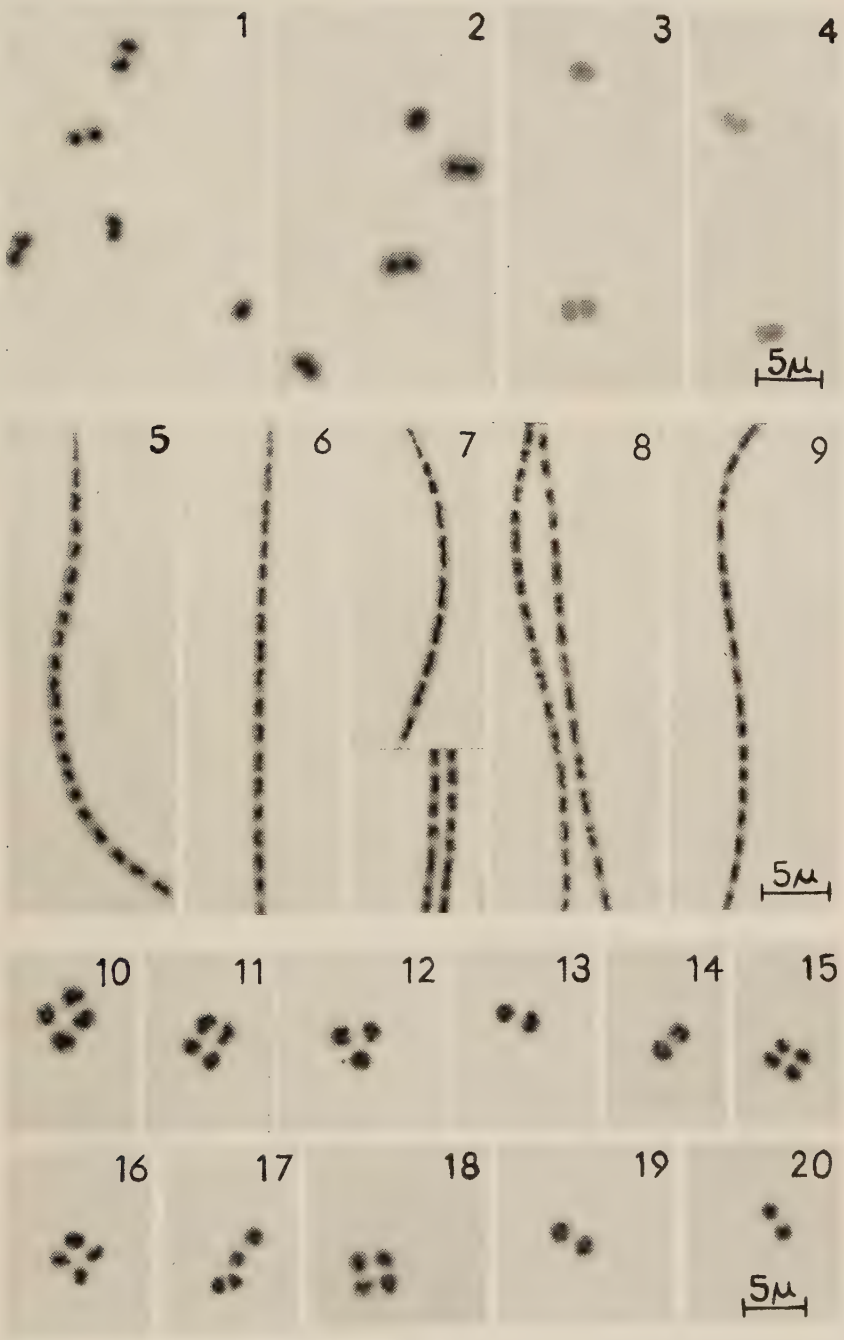
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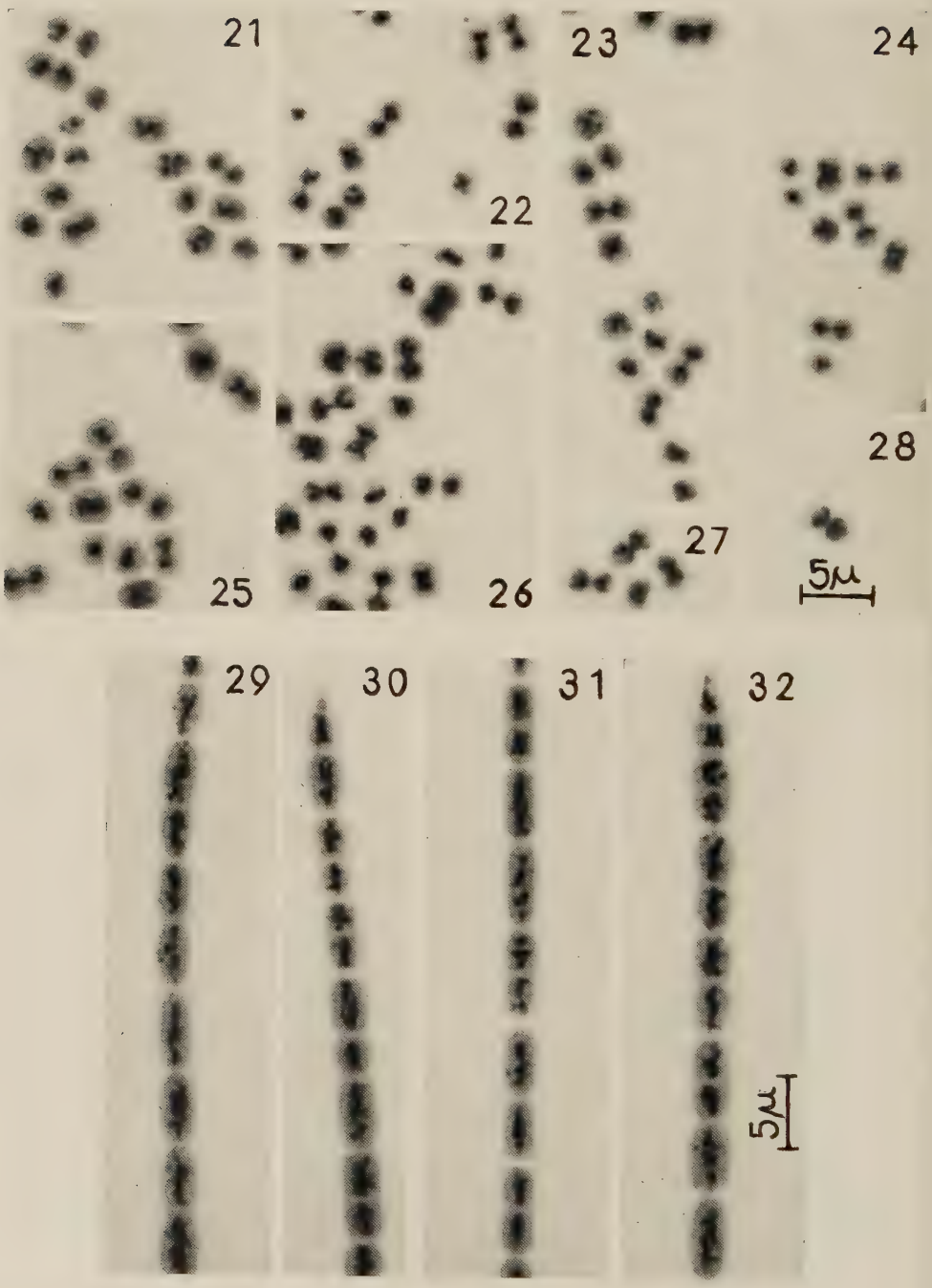
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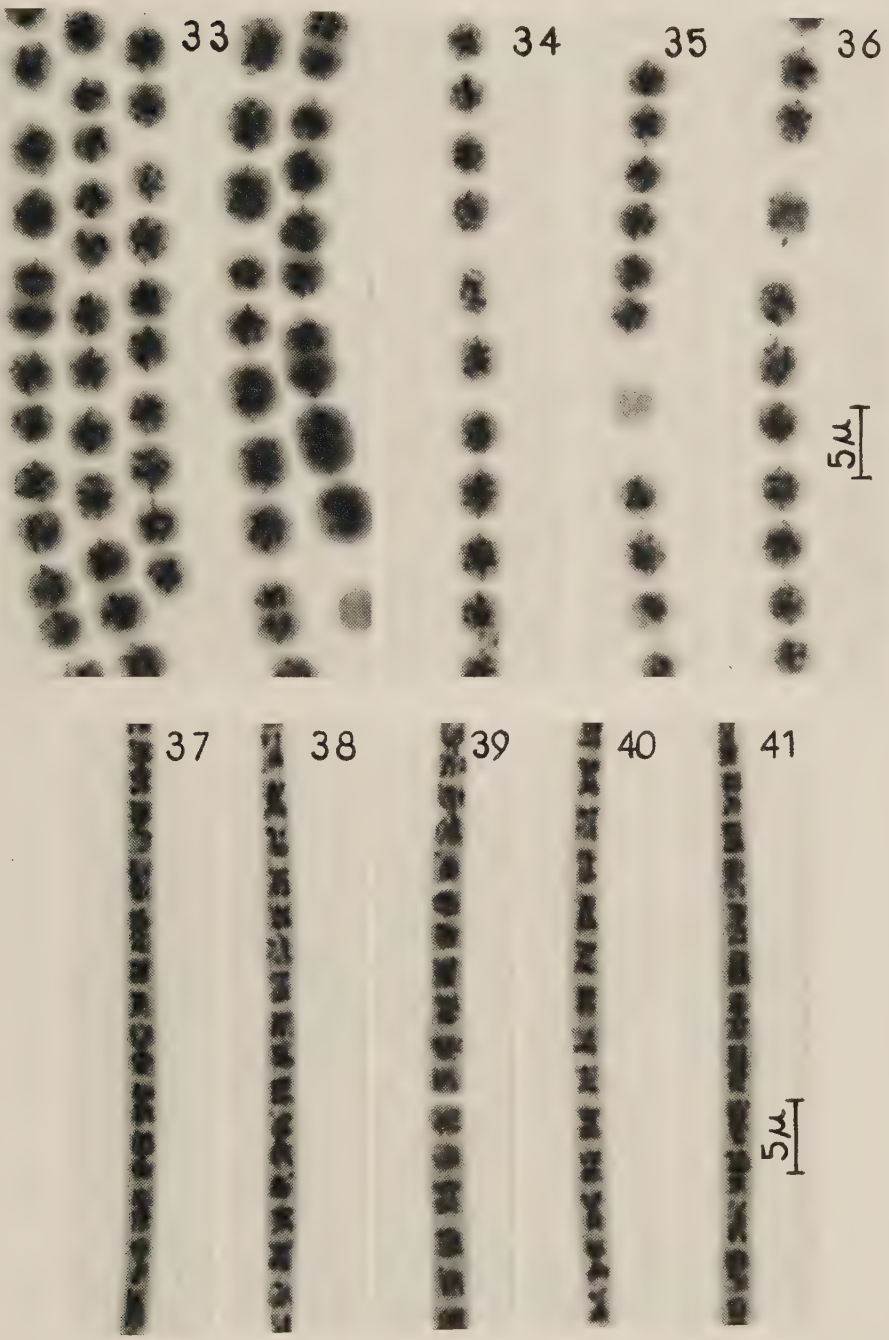
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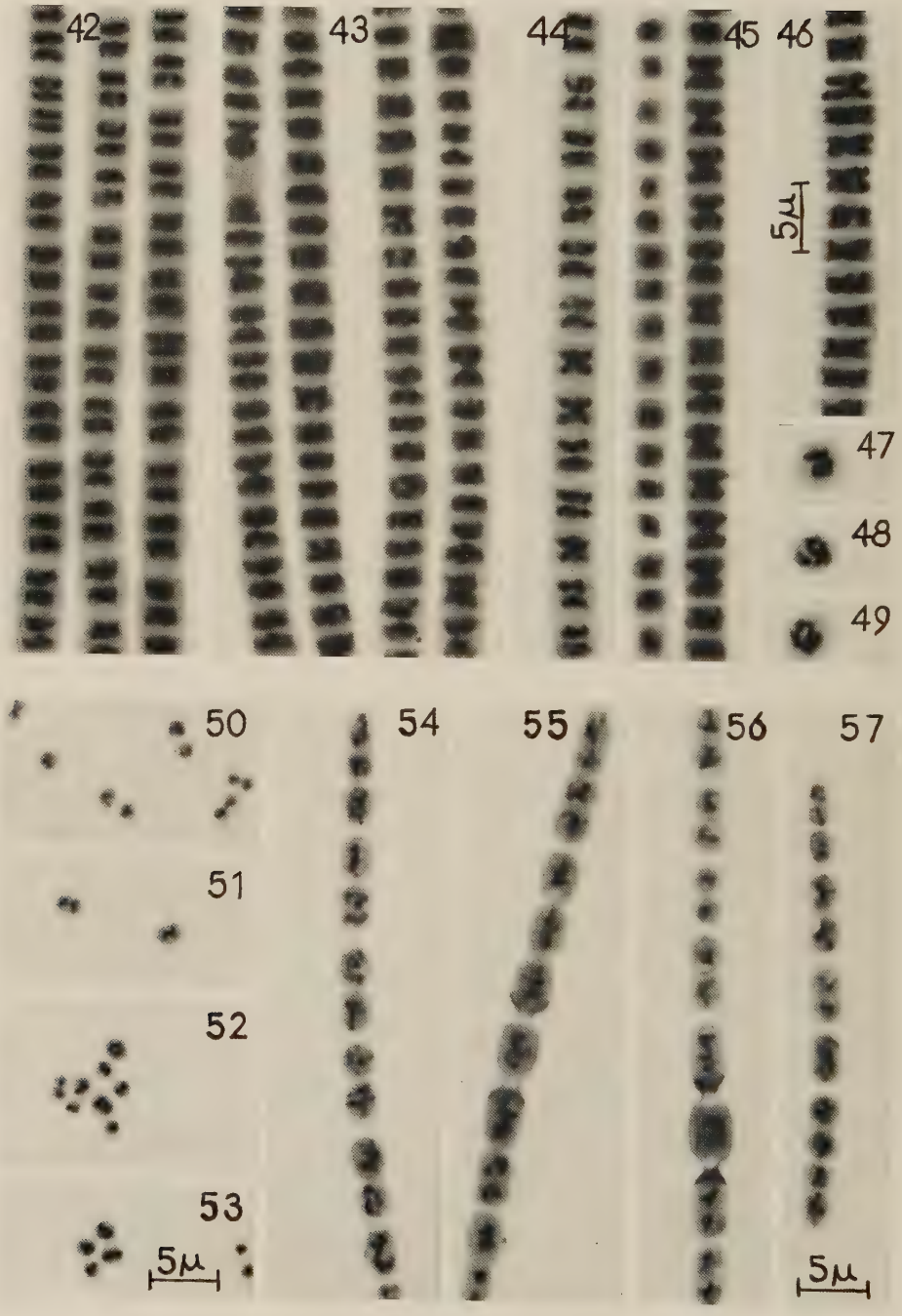
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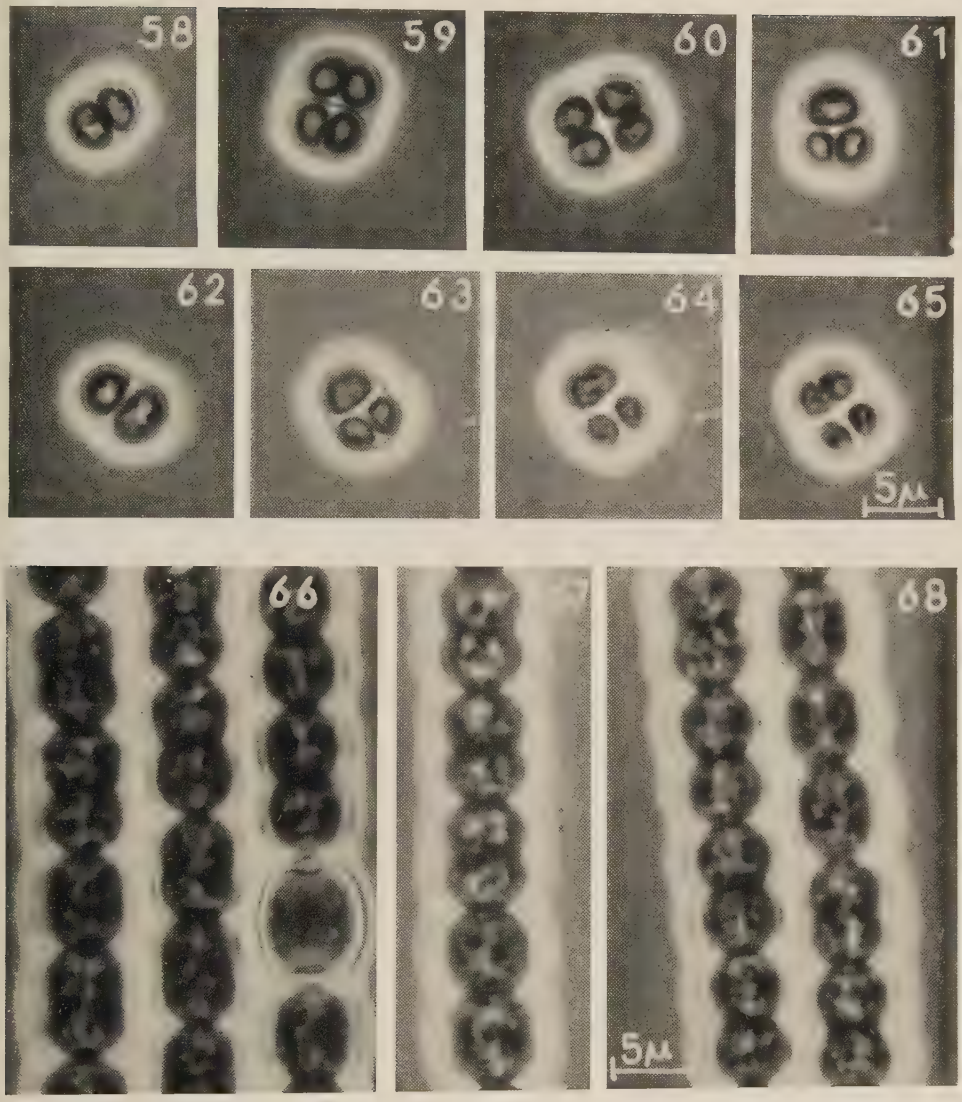
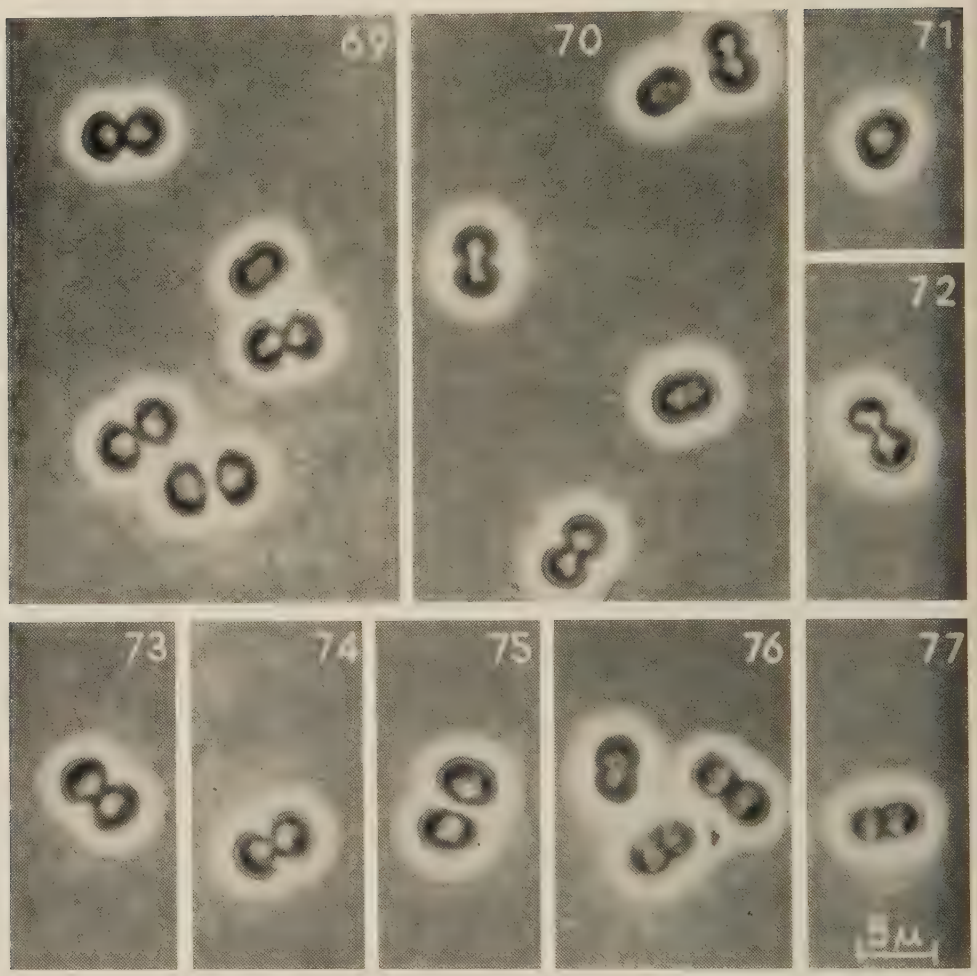
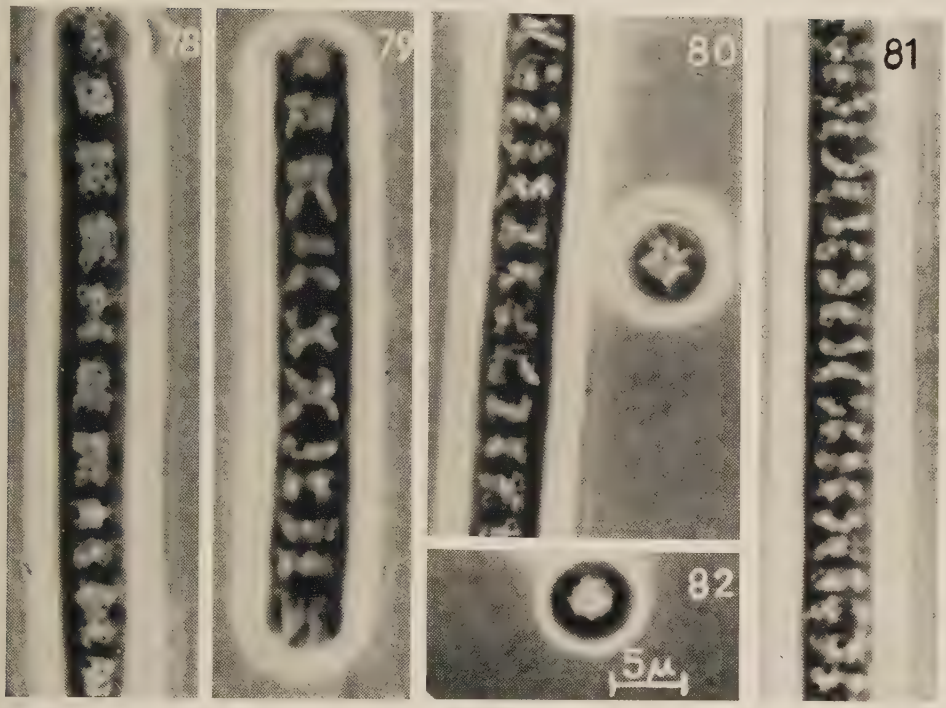


important studies not mentioned are those of Reinhard (42), Dangeard (5), Wager (53), Guilliermond (15), and Neumann (34). The authors of a number of the above reports do not consider the centroplasm to be a nucleus, despite evidence in their studies to the contrary. This lack of agreement between evidence and conclusion is due primarily to the diverse definitions of a nucleus. It must also be pointed out that some who believe that a nucleus is



present have obviously mistaken ergastic substances for chromatin. The many reports denying the existence of a nuclear organization are not listed here since they have been reviewed adequately by Fritsch (11).

Although there is some agreement on the organization of the centroplasm, the details of structure remain to be clarified. Because of this, and the fact that many biologists believe that the *Myxophyceae* are anucleate, it seemed desirable to make a further cytological study of these organisms. As pointed out by Spearing (51), the classical methods of cytological investigation have been employed exhaustively by capable investigators and new methods are needed. The similarities between certain *Myxophyceae* and *Schizomycetes*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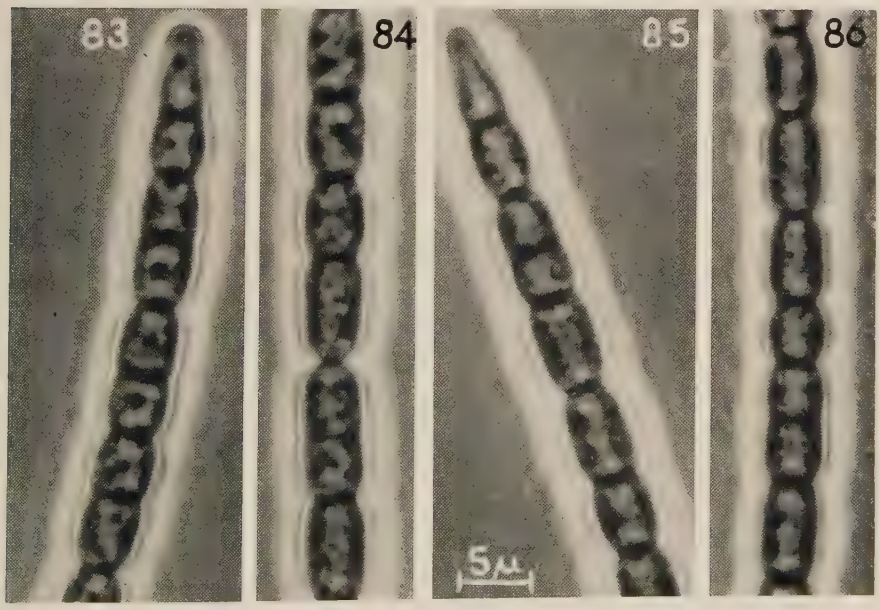
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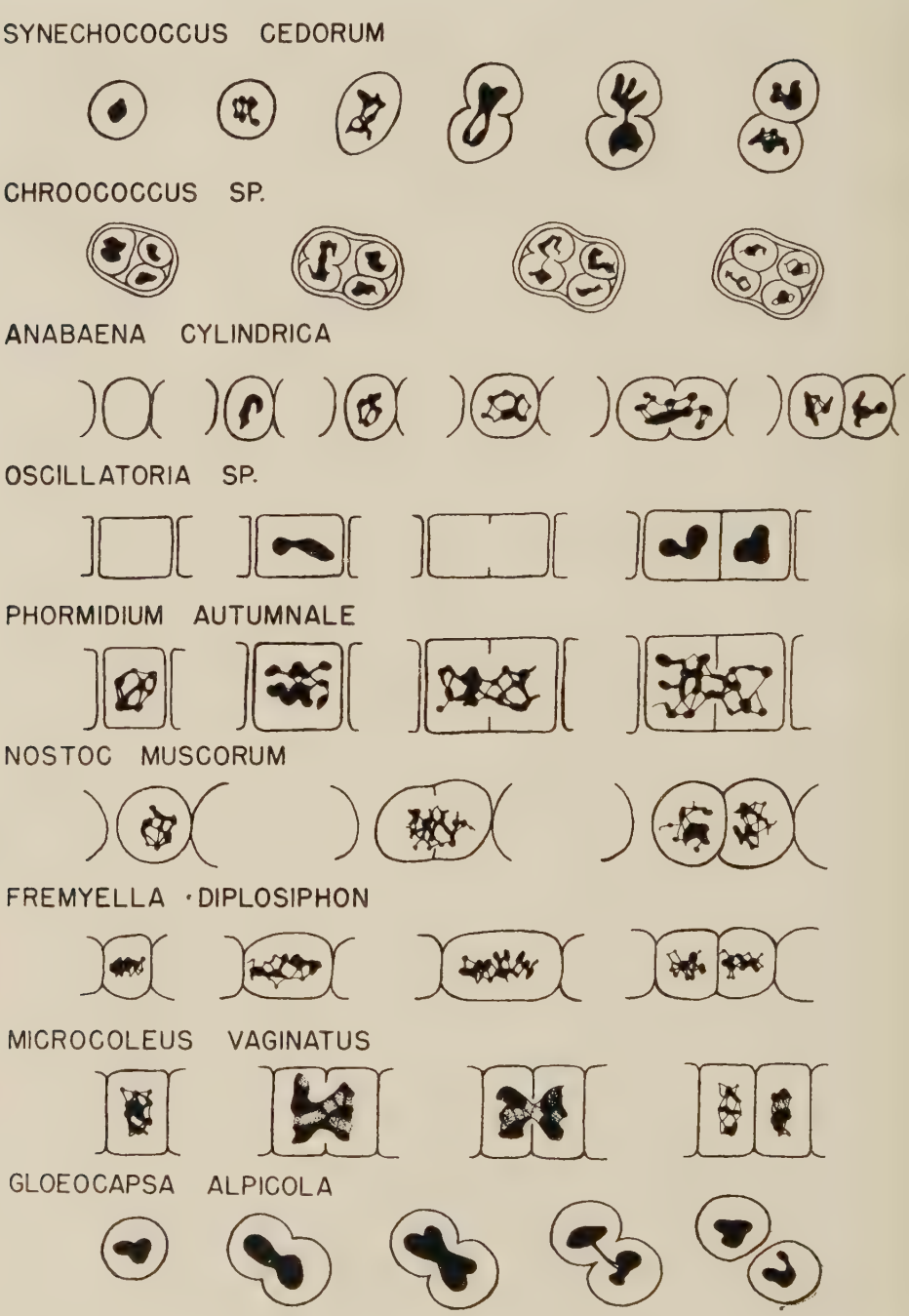
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CHROOCOCCUS SP.
ANABAENA CYLINDRICA
OSCILLATORIA SP.
PHORMIDIUM AUTUMNALE
NOSTOC MUSCORUM
FREMYELLA DIPLOSIPHON
MICROCOLEUS VAGINATUS
GLOEOCAPSA ALPICOLA

suggested the use of recently developed methods which have proved valuable in bacterial cytology.

After this study was begun, the authors were informed by Dr. E. G. Pringsheim that Dr. C. F. Robinow, employing the same general staining method used in the present work, had made a brief study of the *Myxophyceae*. A photograph of his results with *Nostoc* appears in Ordal's revision of Henrici's book *The Biology of Bacteria* (20). Another study recently brought to the authors' attention is an unpublished report by Dr. J. C. Strickland of the University of Richmond. In this report, presented to the Biology Section of the Virginia Academy of Science in May, 1948, "the results of the application of a smearing technique are presented and some notes on the structure of the chromatin are discussed. The position is taken that the central body of the *Myxophyceae* is equivalent to a nucleus and the included chromatin is chemically and morphologically similar to the chromosomes of the higher plants." According to Dr. Strickland the photographs in the present paper show the typical configurations which he observed.

METHODS

For the preparation of a liquid culture medium, the following quantities of sterile salt solutions were added to 195 ml of pyrex-double-distilled, sterile water; the volume was adjusted to 200 ml: 1 ml 1 per cent $CaCl_2$, 1 ml 5 per cent KNO_3, 0.1 ml 0.5 per cent $FeSO \cdot 7\,H_2O$, 1 ml 2 per cent $MgSO_4 \cdot 7\,H_2O$, 0.2 ml micro elements, and 1 ml 2 per cent K_2HPO_4.[1]

The salt solutions were sterilized by autoclaving for 10 minutes after the temperature reached 115° C. However, sterilization of the ferrous sulfate solution was effected by filtration through sintered glass. The salts were added to the water in the order listed above.

The initial pH of the medium is 7.4, but it may rise to 9.0 or higher, depending on the organism grown in it. If an acid reaction is desired after growth, NH_4NO_3 may be substituted for KNO_3.

A modification of Hoagland and Arnon's (21) solution of micro elements was prepared by adding to 100 ml of water: 0.1430 g H_3BO_3, 0.0905 g $MnCl_2 \cdot 4\,H_2O$, 0.0110 g $ZnSO_4 \cdot 7\,H_2O$, and 0.0020 g $H_2MoO_4 \cdot H_2O$.

Following Pascher (36), the total solid content of the medium was maintained at not more than 0.05 per cent; the anhydrous salts total 0.045 per cent. The culture of *Synechococcus*[2] was kindly supplied by Dr. Mary Belle Allen, who informed the authors that it requires glutamate. Sodium glutamate (2 per cent solution) was, therefore, added to the medium to give a concentration of 0.01 per cent.

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[1] Throughout this report percentage expresses mass per volume.

[2] It is the opinion of Dr. Francis Drouet that the organism described herein may be a dissociated form of *Diplocystis* (*Microcystis*) *aeruginosa*.

Cultures were grown in sterilized 250 ml Erlenmeyer flasks, each containing 40 ml of medium. Although the algae were not bacteria-free, aseptic technique was used to prevent contamination by certain fungi. This is especially important when a solid medium is employed.

The cultures were kept at room temperature in a north window. A moist chamber, with a small opening to the air, was helpful in maintaining cultures on solid media.

For the preparation of a solid medium, solidifying agents such as agar, silica gels, and plaster of Paris were studied. Ludox, recommended by Temple (52), seemed very promising, but because of the low concentration of electrolytes in the medium it would not solidify. Though two means of preparing a solid Ludox medium were eventually discovered, the resulting media did not have the advantages of an agar medium. Agar, therefore, was finally selected as a solidifying agent. After pouring a 3.75 per cent solution about 5 mm deep into flat pyrex baking dishes, the solidified agar was cut into blocks approximately 1 cm square which were washed in distilled water at 40°C for a week, with a change of water twice each day. Following the last washing, the agar was melted by autoclaving for 15 minutes after reaching 115°C. It was then cooled to 45°C and neutralized with potassium hydroxide, bromthymol blue being used as an indicator. Finally, it was bottled and again autoclaved, this time for 10 minutes. A 1.5 per cent agar medium was prepared by adding 80 ml of the agar described above and the required salts (4.3 ml) to 115 ml of water, the water and agar being brought to 45°C before mixing.

The organisms studied include: *Chroococcus* sp., *Gloeocapsa alpicola*, *Synechococcus cedorum*, *Oscillatoria* sp., *Phormidium autumnale*, *Microcoleus vaginatus* (in a lyngbyoid condition), *Anabaena cylindrica*, *Nostoc muscorum*, and *Fremyella diplosiphon*.[1]

The preparations of *Gloeocapsa* and *Chroococcus* were made from cultures grown on a solid medium for approximately two weeks, and those of the other organisms from cultures grown in a liquid medium for one to two weeks. The culture medium was always freshly prepared before inoculation.

Vapor from a 2 per cent solution of osmium tetroxide was used as a fixative. This means of fixation has been very useful in bacterial cytology, and was used as early as 1887 by Kunstler (26). Suspensions of cells or filaments were placed on agar blocks resting on filter paper, and the whole covered with a Petri dish top. After all visible water had disappeared from the surfaces, the blocks were placed on slides and transferred for 2 minutes to a Coplin jar containing the osmium tetroxide solution. Following fixation a drop of water was placed on each block and the organisms loosened with a wire loop. The filamentous forms were transferred to a slide and further teased apart in a drop of water. When the preparation had dried, the slide was placed in normal hydrochloric acid at 60°C for 10 minutes. Since the majority of the cells are lost from the slide when unicellular organisms are manipulated in this way, they were processed by centrifugation in 3 ml tubes. The tubes were immediately cooled after the acid treatment and the cells washed 4 times with tap water. The centrifugate was resuspended in a minimal amount of water and used for preparing smears. The procedure of treating fixed cells with hydrochloric acid prior to staining with a basic dye was probably first used by Schumacher (49, 50) and later by Piekarski (39), Robinow (43, 44, 45), and others.

[1] The authors are indebted to Dr. Francis Drouet for identifying most of the organisms.

Following the acid treatment the smears were rinsed with tap water, and stained with a 1:60 dilution of the Giemsa stain (Harleco) in distilled water. Staining for 20 minutes often gave satisfactory results, but consistently good contrast was obtained by staining for 2 hours with the Giemsa stain followed by 10 per cent tannic acid for 10 to 20 minutes. All of the stained organisms in the figures, except in figure 45, were stained by this tannic acid method. Since the preparations were dried before staining, cells of *Synechococcus* were stained and destained without drying to determine whether structural detail could be increased. Such cells, examined in a water mount, showed no advantage over those stained after drying.

For the Feulgen reaction, Schiff's reagent and the rinse fluid were prepared according to the method of Ludford (30). The reagent was completely decolorized by the method of Lillie (29) which consists of adding 1 g of activated charcoal per 100 ml and filtering after 2 minutes. Hydrolysis in normal hydrochloric acid at 60°C for 10 minutes was followed by washing the suspended cells or smeared slides with distilled water only.

Perchloric acid was also employed as a means of detecting desoxypentose nucleic acid (DNA). Its value in quantitative analysis is described by Ogur and Rosen (35), and its cytological application by Erickson *et al.* (10) and Cassel (4). In the perchloric acid studies fixation in 70 per cent alcohol for 1/2 hour was used, and all organisms, including the filamentous ones, were processed by the centrifugation method mentioned above.

A Spencer phase microscope was used to examine cells in the living state, the best results being obtained with a dark contrast-medium objective. The continual movement of some of the filamentous organisms at first presented a photographic problem. This was solved when it was observed that the gliding of a selected filament could be arrested by bringing the 6 volt ribbon filament lamp, employed as the source of illumination, to full intensity for several minutes while keeping the algal filament in the microscopic field.

The photographs were taken on Ansco Isopan film, employing Köhler illumination, a Kodak Wratten B filter No. 58 (green), and a Bausch and Lomb model L camera.

RESULTS

Within the centroplasmic region of the organisms investigated there are elements which are stained by the hydrochloric acid - Giemsa - tannic acid method. The stainability of these bodies, which are Feulgen positive, is not affected by normal perchloric acid at 4°C, even after 45 hours. Subsequent treatment in normal perchloric acid at 70°C for 20 minutes, however, completely removes the stainability. This is clearly shown in *Synechococcus* (Figs. 1 to 4). Both the response to perchloric acid and the clearly positive Feulgen reaction indicate that the elements contain DNA. In preparations of the unicellular organisms the Feulgen-positive elements were found in practically every cell. With the filamentous forms, trichomes in which the cells lacked internal organization were occasionally found, doubtless because

the reagents failed to penetrate to all cells in effective concentration. To separate the intertwining and overlapping filaments is very difficult and there are frequent small clumps in all preparations.

Tests for metachromatin (volutin) applied following the hydrochloric acid step, described in the fixing and staining procedure, gave no positive results. The tests included those of Acton (1), Gardner (12), Meyer (32), Poljansky and Petruschewsky (40), and Spearing (51). Meyer's test is probably the only one which is relatively specific.

It is not the purpose of this report to describe in detail the division and organization of the chromatinic material in each organism studied, but to draw attention to general types of chromatinic organizations. It is convenient to consider the chromatinic material in the *Myxophyceae* as assuming three general arrangements: 1. a loose, sometimes granular, netlike organization; 2. rodlike elements oriented parallel to the longitudinal axis of the cell groups; and 3. a very much condensed organization of various shapes.

The netlike organization was found in *Chroococcus* (Figs. 10 to 14 and 17 to 20), *Synechococcus* (Figs. 21 to 26), *Fremyella* (Figs. 29 to 32), *Nostoc* (Figs. 33 to 36), *Phormidium* (Figs. 37 to 41), *Microcoleus* (Figs. 42 to 49), and *Anabaena* (Figs. 54 to 57). In *Nostoc, Fremyella* and *Phormidium* this appears to be the only type of organization. Oriented rodlike elements are seen in *Synechococcus* (Figs. 21 to 28) and *Microcoleus* (Figs. 42 to 45). Condensed, usually single, elements were found in the centroplasm of *Oscillatoria* (Figs. 5 to 9), *Chroococcus* (Figs. 11, 12, 15 and 16), *Synechococcus* (Figs. 21 to 26), *Microcoleus* (Fig. 45), *Gloeocapsa* (Figs. 50 to 53), and *Anabaena* (Figs. 56 and 57). The spherical and greatly condensed elements in *Microcoleus* (Fig. 45) may not be normal, and are rarely seen. The condensed chromatinic elements in *Oscillatoria* and *Gloeocapsa* remind one of the chromatinic bodies of bacteria; in fact, the former might be mistaken for a species of *Bacillus*. These two organisms are the smallest which were studied, and compare in size with the average bacterium.

Arrangements of the chromatinic elements in the organisms studied are semidiagrammatically represented in the accompanying drawings.[1]

Several of the organisms were examined with the phase microscope. In living cells regions were seen which correspond to those in which chromatinic material is located in stained cells. Detail in the living cells, however, was lacking. No evidence of a distinct membrane around the centroplasm was noted either in living or in stained cells. Living preparations are represented

[1] The authors wish to acknowledge their indebtedness to Dr. Henry Stempen for preparing the drawings.

in the following figures: *Chroococcus* in 58 to 65, *Nostoc* in 66 to 68, *Synechococcus* in 69 to 77, *Microcoleus* in 78 to 81, and *Fremyella* in 83 to 86.

Attempts to follow the division of the centroplasm in living cells met with little success. Some organisms were difficult to study because of constant movement; and others, though not motile, would not develop under the conditions necessary for good observation. In agar block preparations of *Chroococcus,* however, some cells grew and divided, as shown in Figs. 63 to 65. Figure 64 shows the cells after 5 days, Fig. 65 after 7 days. The division of *Chroococcus* under the conditions employed does not seem to be entirely normal, since the centroplasm becomes obscure as division progresses.

DISCUSSION

It is apparent from the morphological and chemical findings that elements consisting of nuclear material are present in the *Myxophyceae.* The morphological findings agree in part with those reported by Bütschli (3), Wager (53, 54), Kohl (24, 25), Phillips (38), Gardner (12), Guilliermond (15, 16, 17, 18, 19), Dehorne (6), Lee (27), Poljansky and Petruschewsky (40), Gavaudan and Gavaudan (13), Spearing (51), and Delaporte (7). It should also be pointed out that the presence of Feulgen-positive material in the *Myxophyceae* has been reported by Poljansky and Petruschewsky (40), Neumann (34), Petter (37), Hollande (22), Guilliermond (19), Gonçalves da Cunha (14), Spearing (51), and Delaporte (7, 8). Recently, Bringmann (2) reported Feulgen-positive bodies in *Lyngbya,* after treating the organism with lanthanum acetate. As previously mentioned, there was no evidence of a distinct membrane around the centroplasm.

The occurrence of chromosomes and mitosis, or a simulation of these, in the *Myxophyceae* has been reported by several investigators, and this matter is fully discussed by Fritsch (11). Although rodlike elements were seen in *Microcoleus* and *Synechococcus* in the present study, the authors are not able to identify them definitely as chromosomes from the present evidence. Reasons for this include the facts that the rodlike elements do not occur consistently at the time of cell division, and that their size and number varies from cell to cell. Also, the connecting thread of material seen between the chromatinic masses at division indicates that independent morphological units are not involved. That these threads are chromosome bridges seems unlikely in view of their occurrence in all dividing cells. Furthermore, the constrictive type of division of the chromatinic material does not suggest mitosis. The authors are inclined to agree with Fritsch (11) that opinions

supporting mitosis are probably the result of misinterpretations. In favor of the opposite view, however, it must be recognized that the technique employed in this study is one which might destroy a mitotic spindle if present. Lewis and Zirkle (28) reported a primitive nuclear division in *Porphyridium*, one of the *Rhodophyceae*. According to these authors, it may be regarded as mitotic, "but the mitosis is quite primitive and of an exceptional kind". The nuclear division is very similar to that described here for the *Myxophyceae*.

Judging from observations on both stained and living cells, quantitatively equal division of the chromatinic material occurs by a constrictive process. This is particularly apparent in *Synechococcus* (Figs. 21 to 28) and in *Microcoleus* (Figs. 43, 45, and 46). What role the centripetally ingrowing cell wall plays in this process is uncertain.

The one substance most likely to be confused with chromatin is metachromatin which is considered as identical with volutin by Meyer (32), Kohl (25), Zacharias (55), Poljansky and Petruschewsky (40), Guilliermond (19), and Fritsch (11). Zuelzer (56) and Prát (41) have reported its disappearance on a phosphorous-deficient medium, while its absence in young cells has been recorded by Elenkin and Danilov (9) and Poljansky and Petruschewsky (40). In addition, the work of Kohl (24) indirectly indicates the absence of metachromatin in young cells. As discussed by Fritsch (11), several investigators have pointed out the solubility of metachromatin in mineral acids, but accurate data on specific concentrations of acids and the duration of exposure to them are lacking. Petter (37) in his work on bacteria indicates that volutin is removed by the acid treatment in the Feulgen reaction, and this property is confirmed by Knaysi (23).

The authors are confident that the hydrochloric acid step in the staining method will remove metachromatin. That chromatin has not been confused with metachromatin is indicated by the negative response to tests for metachromatin, the Feulgen-positive nature of the elements involved, their relatively elaborate organization, and their constant occurrence.

The staining method used here has certain advantages over and above the staining of chromatinic elements. Cyanophycin is undoubtedly removed by the hydrochloric acid treatment, since it is soluble in "dilute" mineral acids; and metachromatin, as previously mentioned, is apparently also removed. In addition, hydrochloric acid has a clearing action on the cells as a whole. The method is simple, apparently specific, and preparations stain well enough to yield satisfactory photographs. Although some detail is lost in photographs they are desirable, since the results of cytological studies on

the *Myxophyceae* have been recorded almost exclusively by drawings. The results, therefore, have tended to reflect individual interpretations.

CONCLUSION

It is concluded that the *Myxophyceae* possess material which is clearly of a nuclear nature; therefore, they should not be regarded as anucleate organisms.

SUMMARY

Several members of the *Myxophyceae* were studied to determine whether or not they possess a nuclear organization. The fixing and staining methods employed are ones which have found extensive use in bacterial cytology in recent years. The Feulgen reaction and differential extraction with perchloric acid both indicate that the elements revealed by staining contain desoxypentose nucleic acid. These elements may be condensed, netlike, or rodlike, and at the time of cell division they appear to divide equally by a constrictive process. What role the centripetally ingrowing cell wall plays in this process is not certain. There is every reason to believe that the elements constitute a nuclear organization.

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Experimental Cell Research, **6**, *151–154 (1954)*

THE FORMATION OF WHITE DEPOSITS IN TISSUE CULTURES IN THE PRESENCE OF ORGANIC PHOSPHATES

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Received June 15, 1953

A FORMATION of white deposits is occasionally seen in the periphery of cultures of tissue cells *in vitro* especially when the cultivation is made in Carrel flasks. These deposits absorb light and appear as brownish rings in transmitted light. Fischer, Laser and Meyer (4) found the deposits to occur more often in cultures of malignant tissues (Ehrlich carcinoma) than in cultures of normal tissue. They found also that an increase in the calcium content of the medium resulted in an increased production of these deposits. The occurrence of acidic metabolic products (lactic acid) was suggested as a possible source of their formation.

We have found that the deposits are produced in cultures of normal tissue when organic phosphates are present.

EXPERIMENTS AND RESULTS

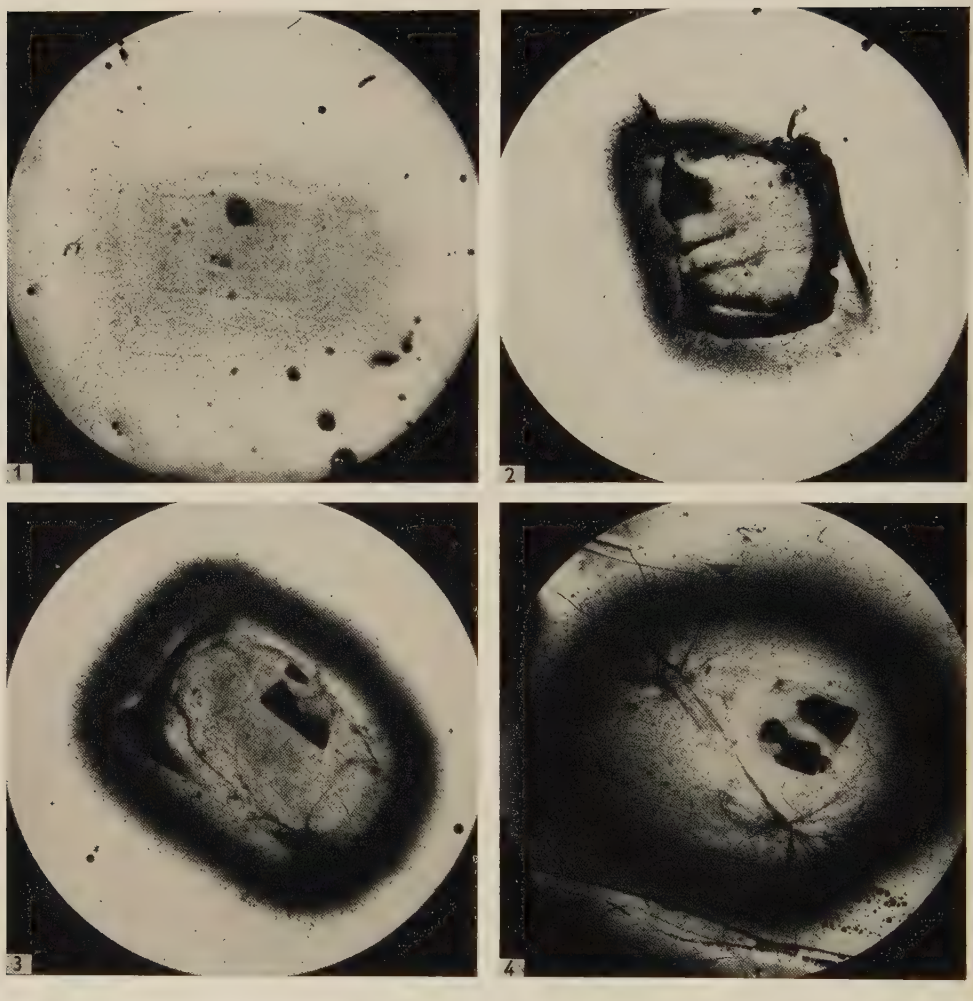
Chick heart fibroblasts cultivated for 5–6 passages on cover slips were used. They were cut into halves and placed in Carrel flasks. The solid phase consisted of 0.5 ml fresh chick plasma and 1.0 ml Ringer solution clotted with one drop of embryonic extract. The fluid phase consisted of 0.4 ml serum, 0.1 ml tyrode solution and 0.2 ml Ringer solution (in the controls) or 0.2 ml of the solution to be investigated (in the experiments).

In some cases the cultures in the solid phase were first treated for one hour with 2 ml tyrode and then left for 24 hours at 39°, again treated with 2 ml tyrode for one hour and the final fluid phase then added. This was done in order to remove soluble components from the media and tissue explants before the experiments started. In other experiments dialyzed serum was added instead of normal serum. This technique is similar to that used by Fischer in his original experiments on the growth of tissue cells in dialyzed media (2).

In the first experiments a solution of the following composition (V-621) was added: 50 mg glutamine, 60 mg fructose-diphosphate (Ca salt) and 40 mg glutathione.

The substances were dissolved in 20 ml 0.9 per cent NaCl and sterile-filtered. The solution is derived from the synthetic mixtures previously described (3).

When 0.2 ml of V-621 were added it was noted that the experimental cultures were covered just inside the periphery of the culture by a white deposit. Further a considerable increase in area over the controls was seen.



Formation of white deposits in cultures of heart fibroblasts. Age: 6 days. Magnification: 6 ×. Living.

Fig. 1. Control. Normal medium. Calcification only in the oldest part of the explant.
Fig. 2. Experiment containing fructose-diphosphate (Ca). Diffuse calcification in part of the periphery. Extensive calcification in the central part and along the margins of the explant.
Fig. 3. Experiment as in Fig. 2. Extensive calcification in the periphery. Calcification of central piece as in Fig. 2.
Fig. 4. Experiment as in Fig. 2. Very extensive calcification in the periphery. Less extensive calcification along the borders of the central piece than seen in Figs. 2 and 3.

In further experiments it was found that the white precipitates were formed when the solution added contained only the fructose-diphosphate (as calcium salt). Neither glutamine alone nor glutathione produced white precipitates. The white deposits appeared as well in cultures containing concentrated embryonic extract as in those containing diluted extract, but the growth of the culture was of course considerably larger in the first case. It was interesting to note that the fructose-diphosphate solution alone produced a larger growth area in the experiments than was found in the control cultures containing no fructose-diphosphate. Glutamine alone or glutathione had only a slight effect on the growth area.

The results of these experiments suggest that the formation of the white precipitates is caused by an enzymatic splitting of fructose-diphosphate by tissue phosphatases. The released phosphoric acid then reacts with calcium ions in the alkaline medium to form a precipitate of calcium phosphate. In that case other organic phosphates should react in the same manner. A number of substances were tested for their ability to form white precipitates in experiments, where a solution of fructose-diphosphate served as control. White deposits were formed when calcium glycerophosphate was used (60 mg in 20 ml solution). When the sodium salt of α-glycerophosphate was used instead of the calcium salt white deposits were still formed, but to a slightly lesser extent. Deposits appeared also, when phosphorylethanolamine was used. (The phosphorylethanolamine was prepared as barium salt according to Outhouse (10)). Barium was removed by means of sulphuric acid and the solution neutralized and used in a concentration corresponding to 60 mg phosphorylethanolamine in 20 ml). No precipitate was formed when calcium lactate (60 mg in 20 ml) or sodium citrate (3.5 per cent, secondary) were used. The appearence of the white deposits is shown in Figs. 1 to 4 in transmitted light.

DISCUSSION

The experiments presented here show that the formation of the white deposits occurs when organic phosphates are present in the cultivation medium (especially as calcium salts). Lactic acid is not involved. This clearly suggests that the formation is caused by the action of a phosphatase on the organic phosphate resulting in the formation of calcium phosphate deposits. Recent investigations on phosphatases in tissue cultures supported this conclusion (7). Though the growth of the cultures was influenced by the concentration of embryonic extract the formation of the precipitates took place in the presence of diluted as well as concentrated extract. This means that the substances contained in the embryonic extracts are not essential for the formation of the precipitate and that an interaction with cellular substances derived from the tissue is involved. This follows also from the localisation of the deposits. They appear always in the periphery of the tissue culture.

The observation by Fischer *et al.* (4) that the precipitate was produced especially in cultures of carcinoma tissue may reflect an accumulation of

organic phosphates in the metabolism of such tissues. Demuth (1) added calcium glycerophosphate and fructosediphosphate to growing cartilage in cultures, but does not describe any effect similar to that here mentioned. Rapid calcification of cartilage *in vitro* in the presence of organic phosphates has recently been described by Miller, Waldman and McLean (8). Hanks (5) mentioned that heating of embryo juice at 60° for 30 mins. destroys phosphatases and prevents the liberation of excess phosphates which darken the growing cultures. This is a phenomenon reminiscent of ours, but we have found no particular effect of the embryo juice on the development of the deposits. The source of phosphatase in our case is the tissue cells. Later Hanks (6) suggested the action of phosphatases from the cells as a possible explanation. The calcium phosphate deposit studied here appears, as mentioned, at the periphery of the tissue culture, and differs from the calcification taking place in the central piece of the culture, compare Figs. 1–4. The latter deposits show a more solid appearence and have less diffuse boundaries. They have been studied by Möllendorf (9).

SUMMARY

The formation of white deposits in tissue cultures is caused by an interaction between organic phosphates and the tissue, most probably by means of phosphatases released from the cells.

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Experimental Cell Research, **6**, *155–161 (1954)*

CUTTING OF SECTIONS FOR ELECTRON MICROSCOPY WITH A MODIFIED "CAMBRIDGE" ROCKING MICROTOME

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Received June 20, 1953

THE need in electron microscopy for tissue sections of the order of 0.1 μ thickness has led to the development of suitable microtomes along two different lines: (a) the construction of new types of microtome such as the high-speed microtome (4) and others (3, 11); and (b) the modification of existing microtomes. The second line has led to such instruments as the modified Spencer Rotary Microtome (9) and the modified Cambridge Rocking Microtome (1).

The latter instrument was produced by merely altering certain dimensions in the instrument such as screw-pitch, number of teeth per unit angle on the wheel, etc. We have tried to determine the limit to which it is possible to carry this process, and to discover whether it is imposed by the impossibility of cutting sections below a certain thickness or by the impossibility of further reduction of the dimensions of the instrument.

EXPERIMENTAL

Without modifying the base of a standard Cambridge Rocking Microtome there are three modifications which can be made to reduce the advance:

(i) Reduction of the pitch of the screw-column. (ii) Increase of the total number of teeth on the toothed wheel. (iii) Decrease of the height/length ratio of the advance arm.

An instrument was made in which these alterations were, in the opinion of a firm of precision engineers, taken to the limit compatible with efficient working. The final dimensions were:

Pitch of screw 1/92″; number of teeth on wheel 500; height/length ratio 1/24. Fig. 1 illustrates the modified arm.

The corresponding dimensions for the unmodified instrument were 1/26″, 330 and 1/6, and the minimum advance was 0.5 μ. The theoretical minimum advance on the present instrument is therefore $0.5 \times \frac{26}{92} \times \frac{330}{500} \times \frac{1}{4} \simeq 0.25$ μ.

With a modified screw only the limit is 0.14 μ; with a modified screw and wheel the minimum advance is just less than 0.1 μ, and sections were cut at this thickness with ease. The commercial rocking microtome for cutting sections for electron microscopy fitted with such a screw and wheel gave sections of nominal thickness 0.05 μ which, as described below, could be cut considerably thinner than this.

By reason of the general design of the instrument, the modified arm could be fitted with ease only to the older type of standard microtome. When so fitted, it produces, as shown above, a minimum advance of about 0.025 μ, and serial sections were cut at this thickness. The details of the fixing, embedding and cutting are given below.

The reduction of the height of the advance arm requires, of course, a corresponding reduction in the height of the cutting edge and a shorter spring for the rocker arm. Thus modified, the microtome works perfectly well.

Cutting very thin sections with the modified "Cambridge" Rocking Microtome. The tissue to be examined was first cut into very small pieces (1 mm^3 or less) with a safety razor blade, the tissue being wet with the fixing solution during cutting. The fixative used was 2 per cent osmium tetroxide dissolved in M/30 veronal-acetate buffer at pH 7.35; Palade (8) who recommended this buffer, found that 1 per cent osmium tetroxide was sufficient. We found that 2 per cent osmium tetroxide gave more consistently good fixation, al-

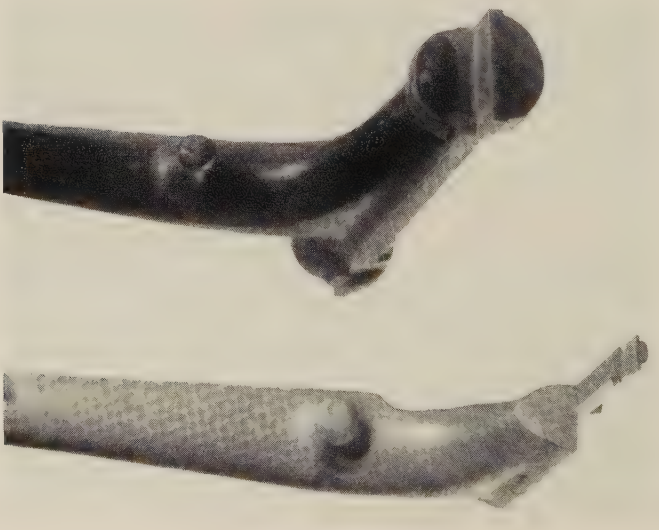

Fig. 1. The old (above) and new advance arms. The apparent ratio of their heights has been altered, by photography, to just over 3.

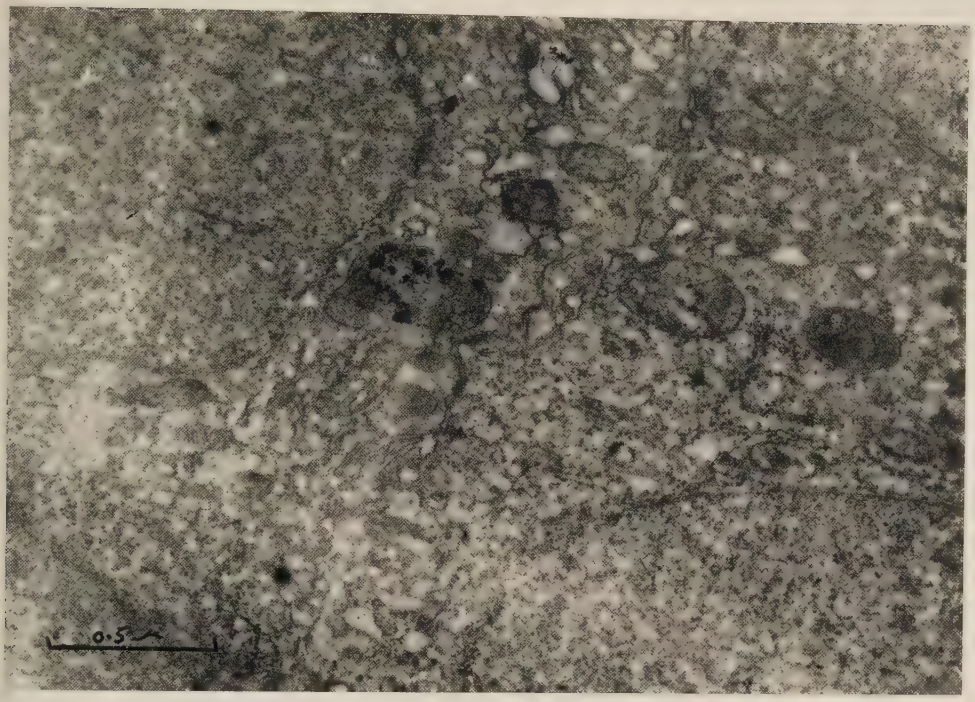

Fig. 2. C3H mouse breast tumour. A general view showing parts of three nuclei and a mass of cytoplasm.

though 1 per cent was often adequate. Pieces of solid tissue were fixed for 3–4 hours. Chorio-allantoic membranes of developing eggs were "dropped" the day before they were to be fixed, to allow the chorionic cells to recover from the trauma involved. A few drops of the fixative were placed on the chorionic surface and 1 ml of fixative was injected into the allantoic cavity beneath the piece to be removed. Fifteen minutes later, pieces of membrane 5 mm sq. were cut out of the egg with scissors and placed for a further two hours in the fixative. Where amniotic membrane was also to be fixed, 0.5 ml of fixative was injected into the amniotic cavity as well.

After dehydration in 70, 90 per cent, and 2 changes of absolute alcohol, the tissue was soaked in 3 changes of a mixture of 1 part n-methyl methacrylate to 3 parts n-butyl methacrylate from which the inhibitor had been removed and to which 1 per cent w/v benzoyl peroxide had been added; for polymerisation to occur the tissue was then placed in the monomer mixture in gelatin capsules at 47°, as originally described by Newman, Borysko and Swerdlow (6).

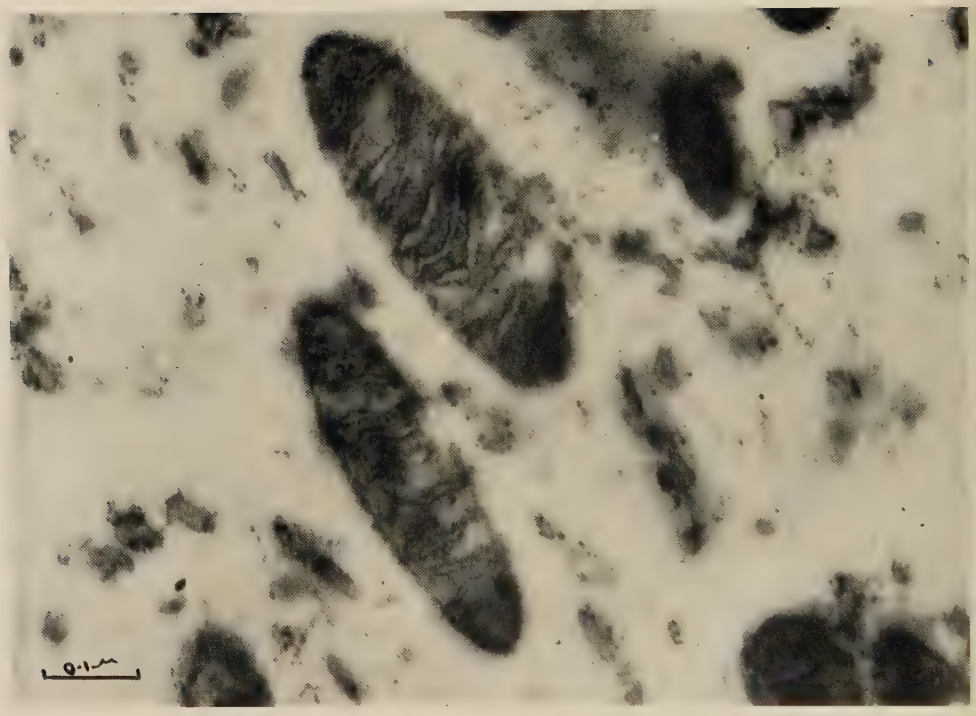

Fig. 3. Mouse kidney. Two mitochondria, showing peripheral and transverse double membranes.

We found that pure methyl methacrylate was so hard that although good sections could be cut, the glass edge was easily damaged. Pure butyl methacrylate was too soft; it was easily cut, but sections were uneven in thickness when cut at less than 0.1 μ. A 1 : 2 or 1 : 3 mixture gave the best results.

The inhibitor (0.1 per cent hydroquinone) was removed from the monomer before use by extraction with 10 per cent aqueous potassium hydroxide, washing with phosphate buffer pH 6.5, and several changes of distilled water. The monomer was mixed with the benzoyl peroxide, dried with anhydrous calcium chloride, and filtered.

The monomer will polymerize quite well if the inhibitor is not removed; presumably the benzoyl peroxide oxidizes the hydroquinone, but the time taken for polymerization is much more variable than when the inhibitor has been removed. Furthermore, if the inhibitor is not removed, the tissue usually appears damaged by the polymerization.

The plastic blocks were trimmed with a scalpel and sealed into the specimen holder of the microtome with diethylene glycol distearate (the main

component of "ester wax"). This had the advantage of being hard and rigid and having a low melting point. The thinner the section to be cut, the narrower it had to be. For a section of 250 Å thick, the block was usually trimmed to about 0.3 mm × 0.7 mm, the smaller dimension always facing the knife. Sections of a uniform (as judged by interference colour) thickness of 0.05 μ could be cut from a block face of 0.75 × 1.2 mm.

Sections 0.1 μ thick had a bluish violet interference colour by reflected light; sections 0.05–0.06 μ thick uneven orange-yellow; when thinner than this they appeared colourless.

Sections were cut on glass knives (5). Pieces of broken glass were held between two suitably shaped pieces of brass, clamped together with a G-clamp.

Acknowledgment is due to Messrs. Chance Bros. for a suggestion that glass of high silica content might yield an edge less liable to chip during cutting. Although there was no appreciable difference in this respect from ordinary glass, 'Hysil' glass was found to retain its edge better than softer glass and to give better sections. This may be due to its higher thermal softening point; it is likely that a high temperature is reached at the cutting edge, because thin sections, even of pure methyl methacrylate polymer (or commercial perspex) adhered firmly to each other to form ribbons, and the plastic could sometimes be seen in the electron microscope to have fused at the junction of two sections.

As the sections were cut, they were collected on the surface of a mixture of acetone, 1 part, and water, 3 parts. Cutting was watched through a binocular dissecting microscope at a magnification of × 30. Sections were best cut slowly; alternate slow and quick cuts, especially with large block faces, sometimes gave alternate thick and thin sections; in this way sections thin enough to give very good resolution could be cut even with a microtome set to an advance of 0.05 μ.

It frequently happened that when the liquid in the trough came up to the knife edge, the section just cut was picked up by the ascending block of plastic. Two ways were found of overcoming this: (i) Several sections could be cut dry, and then when the liquid level was raised, they flattened out into a ribbon. (ii) Sections could be detached from the edge as soon as they were cut by the use of a needle or the point of a No. 7 watchmaker's forceps.

However, the ideal solution to this problem lies in the use of a microtome in which the tissue block moves past the cutting edge in one direction only.

The water trough attached to the knife was of plasticine.

Elaborate precautions against vibration were found to be unnecessary with

the rocking microtome, probably because its only sliding parts are the handle and the spindle carrying the screw. No "backlash" could be observed. Sections could be cut at room temperature (18–20°), but variation in temperature had to be guarded against.

Some sections varied in thickness in different areas. The thinner areas gave the best resolution although resolutions of 30 Å could be obtained everywhere.

Sections were mounted on the thinnest Formvar membranes which would remain intact; a 0.1 per cent solution in chloroform was poured on to the slides and quickly allowed to drain off; the resulting membranes were satisfactory.

Observations were made with an RCA EMU 2C electron microscope, fitted with a 70 μ objective aperture. The visual contrast of the specimens in the electron microscope, as seen on the fluorescent screen, was low. To get exact focus, through-focal series of photographs were taken at a magnification of 22,000.

Some typical results. Pieces of mouse kidney, mouse lactating mammary gland and C3H carcinoma, and of chorio-allantoic and amniotic membranes of chick embryos of 12 days' incubation were fixed and embedded as described earlier, in some cases without removing the inhibitor from the methacrylate monomer. Sections were cut at an estimated thickness of 250 Å.

Some typical results are shown in Figs. 2 and 3. They are exhibited only as specimens of the sections obtained and will be discussed in more detail elsewhere. Fig. 2 shows a general view of a field. Clearly visible are nuclei and various cytoplasmic structures. The structure of the mitochondria is shown.

Fig. 3 represents a pair of mitochondria. The structure of the peripheral and transverse walls is clearly visible.

These results confirm those obtained by other workers (7, 10).

DISCUSSION OF RESULTS

The thickness of the sections. So far no direct evidence of the actual thickness of the sections is available, although arrangements have been made with Professor S. Tolansky, of the Department of Physics, The Royal Technical College, Holloway, to try to determine the thickness by interferometric methods. The evidence on which the mean thickness of the sections is stated to be about 0.025 μ is as follows:—

(i) The dimensions of the instrument are such that the calculated mini-

CORRECTION

EAVES, G., and FLEWETT, T. H., *Exptl. Cell Research*, **6**, 161 (1954), line 3: "There are thus, by the theory of Newton's colour scale (10)", should read "(2)".

+ supporting film must therefore be less than 300 Å.

SUMMARY

A modification of the standard Cambridge Rocking Microtome is described, by means of which sections 0.02 μ thick can be cut.

A suitable technique of fixing and embedding is described, and some results obtained are shown.

Thanks are due to Dr. L. L. Dmochowski for the provision of some of the specimens used.

Acknowledgments are also due to Messrs. Barber and Duffy, for making the modified lever and screw; to the Cambridge Instrument Co. for the modification to the wheel; to Mr. C. N. England for photographs of the instrument, and to Mr. J. B. Kirkham for the enlargements of the micrographs.

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LARGE-SCALE CONSTRICTION AND SEGREGATION OF POLAR LOBES FROM THE EGGS OF *MYTILUS EDULIS*

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Received June 30, 1953

The development of isolated blastomeres and egg fragments demonstrates that in the uncleaved eggs of many animals there are cytoplasmic regions which are essential for the differentiation of specific parts of the embryo. One of the most striking examples of this is the vegetative cytoplasm in the mosaic eggs of several annelids and molluscs (*Dentalium, Sabellaria, Chaetopterus* and others). This vegetative cytoplasm, variously called the polar, antipolar or yolk lobe, becomes conspicuous as a bulge at the vegetal pole of the egg shortly before the first cleavage. At the close of cleavage it fuses with one of the cleavage blastomeres. Removal of the polar lobe leads to various defects of larval organization (4, 6, 12). The eggs of *Mytilus edulis,* used in the following experiments, similarly form polar lobes prior to cleavage (5) and removal of the polar lobe cytoplasm causes larval defects (11).

Attempts to study the chemical nature of the polar lobe cytoplasm as well as other regions of mosaic eggs, have consisted mainly of cytochemical investigations, which, although revealing a degree of chemical heterogeneity, are rather limited in scope (3). The techniques to be described were developed to obtain sufficient amounts of cytoplasm to carry out quantitative physiological and biochemical studies.

In an earlier investigation of the respiration of polar lobe cytoplasm (2), isolated polar lobes were obtained in hypertonic solutions of $MgCl_2$. Constriction of the polar lobes from the eggs was thought to be a specific effect ofMg ions; however, the following experiments demonstrate that hypertonic solutions of a variety of substances are equally effective.

CONSTRICTION OF POLAR LOBES FROM EGGS IN HYPERTONIC SOLUTIONS

The gametes of *Mytilus edulis* were obtained by allowing the animals to spawn in individual bowls of sea water at room temperature. Eggs from several females were pooled, fertilized and the vitelline membranes removed with a membrane

lysin obtained from sperm (1). After removal of the membranes the eggs were transferred through several dishes of Ca-free sea water. Shortly before cleavage, approximately at the beginning of polar lobe formation, the eggs were placed in the series of hypertonic solutions.

As will be pointed out, hypertonic solutions of a variety of substances will cause constriction of polar lobes from eggs. In general, the effects of these solutions on polar lobe formation and cleavage are similar. The following is a description of the effects of hypertonic NaCl solutions.

Since the optimum degree of hypertonicity for constriction of the polar lobes varies from one batch of eggs to the next, a series of dilutions of a stock NaCl solution was used. In a typical experiment fertilized eggs, with the vitelline membranes removed, were washed in Ca-free sea water and transferred at the first appearance of the polar lobes to a series of NaCl solutions ranging from 0.57 to 0.70 M.

Cleavage was delayed slightly in 0.57 M NaCl. However, normal polar lobes appeared and eventually fused with CD cells. In 0.60 M NaCl cleavage was also delayed and the polar lobes, at the close of cleavage, remained connected to the CD cells by a stalk of cytoplasm which was thinner than normal. Fusion of polar lobes with CD cells occurred in many eggs; however, in others the stalk gradually narrowed and finally disappeared resulting in complete separation of the polar lobe from the CD cell. Occasionally an almost invisible strand of cytoplasm remained which could be broken by slight agitation of the eggs. In 0.63 M NaCl, which was usually found to be the optimum concentration, 50–75 per cent of the polar lobes separated rather than fusing with CD cells. In higher concentrations of NaCl, 0.66 M and above, cleavage became progressively inhibited. In spite of complete inhibition of cleavage, polar lobes slowly appeared in many of the uncleaved eggs and eventually became separated as small globules of cytoplasm.

In hypertonic solutions of NaCl the AB and CD blastomeres, at the close of cleavage, round up and become completely separated due to lack of Ca which is necessary for the formation of the intercellular cement. As a result three sizes of cells are present: CD cells, AB cells, and polar lobes. For segregation of polar lobes, as described in the following section, it is important to have as large a size differential as possible between the polar lobes and the cells from which they are to be segregated. Accordingly, it became necessary to develop a hypertonic solution in which the normal adhesion of the AB and CD cells would be maintained without interfering with constriction of the polar lobes. Several combinations of salts were tested for this purpose. The most satisfactory results were obtained with a combination of sea water

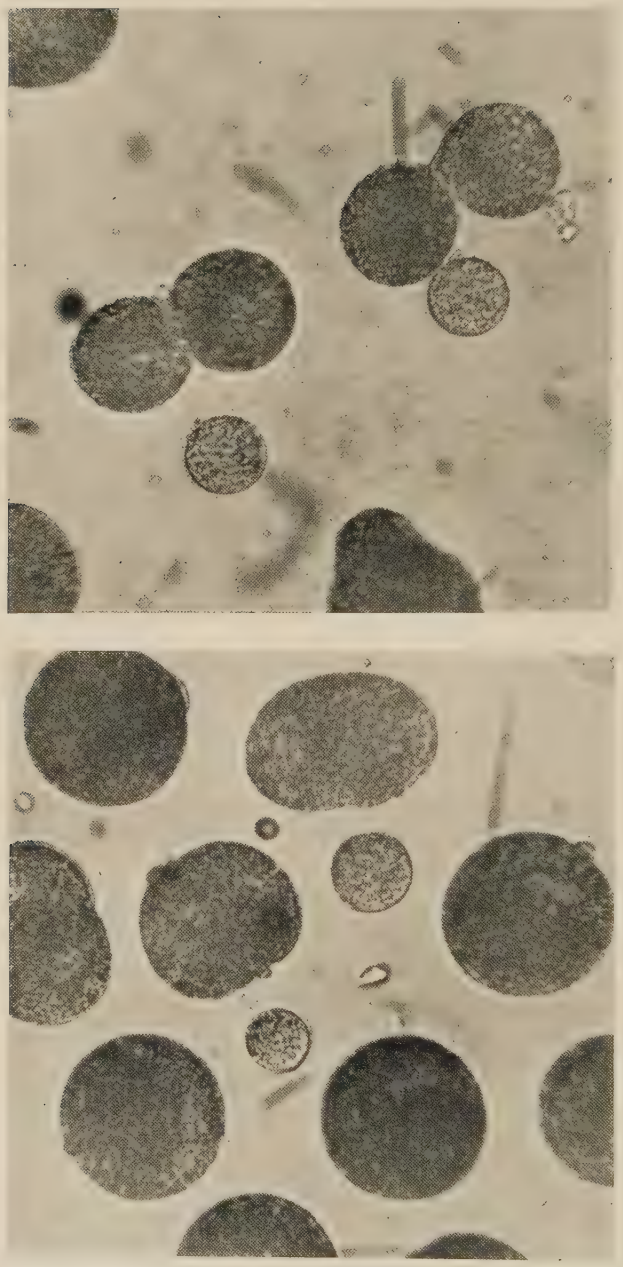

Fig. 1. Effects of a hypertonic solution (NaCl + 25 per cent sea water) on polar lobe formation in eggs of *Mytilus edulis: a*, constriction of polar lobes from CD blastomeres; *b*, constriction of polar lobes from whole eggs.

and NaCl in sufficient amounts (3.3 g NaCl in 100 ml 25 per cent sea water) to permit adherence of blastomeres without preventing polar lobe constriction.

Aside from the adhesion of the blastomeres the effects of this solution on cleavage and polar lobe formation are the same as described for pure NaCl solutions. With increasing hypertonicity of the solution cleavage is progressively delayed and inhibited. In concentrations which inhibit cleavage the polar lobes are constricted from whole eggs (Fig. 1 b); in lower concentration where cleavage still occurs they become separated from CD cells (Fig. 1 a).

In attempts to improve the yield of isolated polar lobes in the above hypertonic solutions, the two variables, temperature and time of transfer, were investigated. The time before cleavage at which the eggs are added to the hypertonic solution does not greatly influence the yield of separated polar lobes. However, if the eggs are transferred too early (20–30 min. before cleavage), the delay and inhibition of cleavage cause a reduction in the number of isolated polar lobes. The optimum time of transfer seems to be at the first appearance of the polar lobe (10–15 min. before cleavage).

In the range from 12° to 20°C there is little effect of temperature on the yield of isolated polar lobes, although it was noticed that the volume of the individual polar lobe tends to increase at lower temperatures. Thus, at 20°C the polar lobes average slightly more than one half the diameter of the CD blastomere, whereas at 12°C they often are nearly as large as the CD blastomere.

Constriction of polar lobes from eggs in hypertonic solutions was developed mainly as a technique for obtaining large amounts of polar lobe cytoplasm. Little has been done to determine the basic causes of the phenomenon, although some general conclusions may be drawn. The constriction of polar lobes in hypertonic solutions of various substances indicates that it is not a specific effect of any particular ion. In addition to NaCl, KCl, $MgCl_2$, LiCl, sucrose, glucose, glycine, and urea, in the proper range of hypertonicity, will cause varying degrees of constriction. Some of the above substances, such as urea or glycine, also cause considerable blister formation and cytolysis which tend to obscure the effects on the polar lobes. Concentrated sea water or $CaCl_2$ are not effective, probably because the intercellular cement is not dissolved and the polar lobes adhere to the eggs.

Separation of polar lobe cytoplasm from eggs by such a wide variety of substances indicates that the basic cause is a dehydration of the cytoplasm. In general, constriction of polar lobes does not occur until the hypertonicity

of any substances attains such a value as to cause a 10 per cent decrease in egg diameter.

Although cytoplasmic dehydration may be considered as a general cause of constriction of polar lobes, there are indications of specific effects of ions. Thus, hypertonic solutions of NaCl or $MgCl_2$ are much more effective than KCl or LiCl. This may be related to secondary effects of these ions on the viscosity of the internal and cortical cytoplasm. Differences in the effects of cations on the viscosity of egg cytoplasm are well known (7).

SEGREGATION OF POLAR LOBES FROM WHOLE EGGS AND CLEAVAGE STAGES

The extremely small volume (approximately 10^{-5} mm^3) of a polar lobe isolated by the previously described method prohibits, at least with the presently available microtechniques, quantitative biochemical or physiological studies on individual polar lobes. It is possible to segregate several hundred of the polar lobes by selection with a micropipette, as was done for measurements of O_2 uptake (2); however, the total amount of cytoplasm obtained by this tedious technique is still very minute. A solution to this problem would be automatic and large scale segregation of the polar lobes from the remaining cells. Since there are no detectable differences in density, such a method would have to be based on the size differences of the cells. The difficulty of the problem becomes evident when one considers the small sizes of the cells; polar lobes, AB cells, CD cells, and whole eggs have diameters of 20–30, 42, 52, and 60 microns respectively.

Large scale segregation was first attempted by construction of wire sieves with spaces between the wires intermediate between the average diameters of isolated polar lobes and the remaining cells. Brass frames, one inch square, were wound with 300–400 turns of 0.001 mil. tungsten wire with spacings of 35 ± 2 microns between the wires. It was expected that on addition of a mixture of whole eggs, polar lobes, AB, and CD cells, the polar lobes would filter through the openings and the larger cells would be retained. In actual practice, however, the yield of segregated polar lobes was disappointingly small. The larger cells, in addition to tending to squeeze through the sieves, would obstruct the openings so that only a small fraction of the polar lobes actually became segregated.

Wire sieves with spacings of 45 ± 2 microns were also constructed for segregation of AB and CD blastomeres. Even less satisfactory results were obtained owing to the small size differences of these cells.

The elutriation technique used for analysis of sediment samples (8), has proven to be more successful for large scale segregation of cells. This method is based on the fact that spherically shaped particles of similar density fall in a fluid at rates which are related to the radii of the particles (Stokes' law).

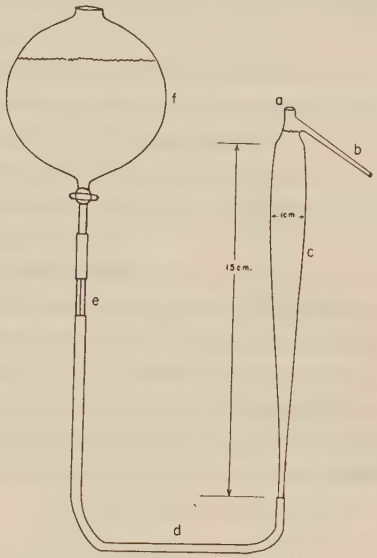

Fig. 2. Elutriator for segregation of polar lobes from eggs and cleavage stages of *Mytilus edulis:* A, opening to introduce cells; B, overflow; C, elutriator tube; D, rubber tubing; E, capillary; F, reservoir.

The basic feature of elutriation consists of a rising current of water in which a grading of different size particles occurs according to their different settling velocities. The simplest type of elutriator used in sediment analysis consists of an upright glass tube in which a carefully controlled ascending flow of water carries the smaller particles upwards and out an overflow whereas the larger ones settle to the bottom.

This principle has been adapted for the segregation of polar lobes from eggs. A number of glass elutriators of various dimensions were constructed with tapered inflow regions, overflow tubes, and openings to introduce the cells. Extensive tests have not been carried out to determine the optimum dimensions. Exploration of this method was carried out mainly with one having the dimensions shown in Fig. 2. This elutriator was drawn from glass tubing with an internal diameter of 1 cm. The upper half is of uniform diameter whereas the lower half is tapered to an opening of 1 mm. The taper must be very gradual and uniform to avoid turbulent and unequal flow of water. Near the upper end, which is open, a small glass tube serves as an overflow.

The problems encountered in the use of an elutriator for segregation of cells are mainly related to obtaining uniform flow velocity. Ideally a uniform flow velocity over the entire cross section of the tube should be maintained, but in actual practice this does not occur. Friction between the wall and flowing water causes a lower peripheral flow velocity. This differential flow becomes less significant as the diameter of the tube is increased; however, the advantages of the greater cross-sectional area becomes, beyond a certain point, offset by a greater probability of eddy currents.

Turbulence caused by water entering the system and by temperature differentials presents a troublesome problem. The turbulence caused by the former is solved to some extent by a very gradual taper in the lower inflow portion of the tube. Temperature differentials in the system are largely eliminated by immersing the elutriator in a water bath and ensuring that the entering water has exactly the same temperature as does the system.

Accurate control of flow velocity is obtained by using a separatory funnel as a reservoir from which the water flows through a short length of fine capillary tube (Fig. 2). Small bore rubber tubing connects the reservoir, capillary tubing and elutriator. The height of the reservoir above the elutriator thus may be used to control the flow velocity of water in the system.

The procedure used for testing elutriators for segregation of polar lobes was as follows. All experiments were carried out in a constant temperature room at 18°C. Polar lobes were constricted from eggs and CD cells using the hypertonic NaCl-sea water solution described in the previous section. The resulting mixture of whole eggs, cleavage stages and polar lobes was transferred to 0.01 per cent chloral hydrate in sea water to prevent subsequent cleavage.[1] The temperature of this solution had been previously equalized with that of the elutriator by immersion in a common water bath. The elutriator was two-thirds filled with sea water containing chloral hydrate, and the mixture of cells was introduced through the top opening and stirred until a homogeneous suspension was obtained. Flow of water was started, and the overflow collected in small containers over a period of several hours.

The optimum flow rate, which should be just sufficient to carry the polar lobes upwards, may be roughly calculated from the fall velocity of the polar lobes and the cross section of the tube. For the elutriator illustrated in Fig. 2 the flow velocity amounts to about 15 ml of water per hour. The proper height of the reservoir above the elutriator to provide this flow is determined

[1] The presence of mitotic inhibitors, such as chloral hydrate, would be undesirable for physiological studies of the segregated polar lobes. In future experiments low temperatures, rather than chemical agents, will be used to inhibit cleavage.

in a blank run using a graduated cylinder to collect the overflow for a fixed period of time. It was found that the number of drops per unit time at the overflow tube can be used as a convenient relative index of flow rate.

In actual practice it was found that segregation of polar lobes could be obtained even if the flow of water in the system considerably exceeded the calculated optimum rate. This was accomplished by starting the flow of water with the elutriator only partially filled which thus permitted an initial segregation of cells before the overflow began. By collecting the overflow water in separate containers for 20-minute intervals it was found that, even with the excessive flow rate, the first portions contained a high percentage of polar lobes. As would be expected, the percentage of eggs and cleavage stages progressively increased in the later portions.

The fall velocities of spherical cells are proportional to the square of their radii; for this reason the elutriation technique becomes more effective as the size differences of the cells increase. Thus, polar lobes are more easily segregated from whole eggs than from cleavage stages. In the proper hypertonic solutions, as pointed out in the previous section, polar lobes are often constricted from eggs which do not subsequently cleave. Although this would be an ideal condition for segregation of polar lobes, the yield of polar lobes is much lower from uncleaved eggs. Accordingly, in the majority of experiments the polar lobes were constricted in solutions of a hypertonicity which permitted cleavage with a higher yield of polar lobes.

Even under the best conditions complete segregation of polar lobes was not attained. A small number of eggs and cleavage stages were always found with the segregated polar lobes; however, these could be easily selected and removed with a micropipette. The amount of polar lobe cytoplasm which may be obtained by the elutriation method is limited, of course, mainly by the amount of starting material. No attempt has been made, as yet, to determine the maximum yield of segregated polar lobes which would be practical in routine experiments; however, in test trials it was estimated that several hundred thousand polar lobes were segregated from eggs and cleavage stages.

The decantation method, also used in sediment analysis (8), has been applied to segregation of polar lobes. This, like the elutriation technique, is based on different fall velocities of particles in a fluid; however, the segregation occurs in a stationary column of water. A homogeneous suspension of polar lobes, whole eggs and first cleavage stages was allowed to settle in a stationary column of water. After a period of time at which it was calculated that the whole eggs and cleavage stages had settled to the bottom, the upper half of

the column was decanted. This contained a small number of segregated polar lobes. Although the yield could be increased by repetition, this procedure was not so satisfactory as the elutriation technique described above.

It would be desirable to obtain segregation of isolated AB and CD cells for physiological studies of first cleavage blastomeres; however, this is difficult owing to the small size differential of the diameters (10 microns). Segregation, by elutriation, of AB cells from whole eggs and CD blastomeres has been accomplished in a few experiments, but the method has not been developed as a routine procedure.

Lindahl and Kiessling (10), using a counter-streaming centrifuge (9), obtained segregation of micromeres from the larger macromeres of the 16-cell stage of the sea urchin. The size differential of the cells was less than 10 microns. Although details of the method have not been reported, the counter-streaming centrifuge would seem to be more suitable than elutriators for segregation of cells with slight size differences.

The combination of the two methods, constriction of polar lobes from eggs in hypertonic solutions and a subsequent segregation by elutriation, allows a considerable accumulation of polar lobe cytoplasm. The preliminary experiments indicate that the quantity obtained may be sufficient to permit the characterization of a specific region of a mosaic egg on a level which has hitherto not been possible.

SUMMARY

Polar lobes become constricted from the eggs of *Mytilus edulis* in hypertonic solutions of various monovalent and divalent salts and nonelectrolytes. This technique may be used for obtaining large numbers of isolated polar lobes. The effectiveness of a variety of substances indicates that the basic mechanism is related to a dehydration of the cytoplasm, although there may be secondary effects of specific cations.

The elutriation principle, used for sediment analysis, has been adapted for segregation of polar lobes which have been separated from whole eggs and cleavage stages. The essential feature of this method is an ascending flow of water which causes segregation of cells of different sizes according to their respective fall velocities.

The author was aided in part by grants from the Committee on Growth acting for the American Cancer Society and from the University of California Board of Research.

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UTILIZATION OF $C^{14}O_2$ BY DEVELOPING AMPHIBIAN EMBRYOS, WITH SPECIAL REFERENCE TO REGIONAL INCORPORATION INTO INDIVIDUAL EMBRYOS

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Received July 5, 1953

Radioactive isotopes have been employed recently by various workers as a means of studying embryonic development. Kutsky (12) investigated phosphate metabolism in *Rana pipiens* using P^{32}, Hultin and Wessel (11) found that developing sea urchin eggs readily incorporated $C^{14}O_2$ and Friedberg and Eakin (8) and Eakin, Kutsky and Berg (6) have studied the uptake of amino acids labeled with carbon-14 by the ventral and dorsal lip regions of individual frog embryos. Friedberg and Eakin (8) discovered that the presence of the surface coat (9) of the amphibian embryo did not allow incorporation of labeled glycine after the gastrula stage and they found it necessary to cut embryos in half in order to obtain uptake of the isotope. By using $C^{14}O_2$, which readily diffuses through the vitelline membrane and surface coat of the amphibian embryo, there is no need to cut individual embryos. Another advantage of carbon dioxide lies in the over-all labeling one obtains with this compound.

In preliminary experiments carboxyl labeled sodium acetate (220 000 counts per minute / mg carbon) was injected into the dorsal lymph space of the female South African toad, *Xenopus laevis*. Pituitary extracts were used to induce spawning, but the low level of isotope uptake found in the eggs did not warrant further use of this sample of low activity acetate. Glucose C-14 (8.9 μc) was used in the same manner, but only the acid-soluble fraction gave a sufficiently high level of activity. It appeared that direct incubation of the eggs with the isotope would be a more satisfactory method than maternal injections.

One purpose of this $C^{14}O_2$ investigation was to conduct a time course

[1] Rockefeller Research Fellow, Wenner-Gren Institute, 1950–51.

study of the radioactivity of various chemical fractions during the development of the frog embryo (*Rana temporaria*) from fertilization to an early larval stage. Particular attention was paid to the relative tracer activity of these chemical fractions in reference to various phases of morphogenesis. Individual chemical compounds were not isolated since specific metabolic reactions were not under investigation. Rather, this study was particularly aimed at the question of regional isotope activity in single embryos. The importance of fields or regions of special morphogenetic potency, e.g., dorsal lip or organizer, within individual embryos is well known from the evidence provided by experimental embryology. It is quite conceivable that results obtained from a fractionation of many embryos might not reveal important regional differences existing in single embryos.

MATERIALS AND METHODS

Rana temporaria eggs were obtained by pituitary injection and artificially fertilized. The eggs were cut out of their jelly, washed in CO_2-free double distilled water and divided into lots of 300 normally developing eggs. Each lot of 300 eggs was washed several times with an acid saline solution,[1] suspended in 2 cc of this solution and transferred to a 21 cc volume Carrel flask. Then 3.5 cc of a $Na_2C^{14}O_3$ solution was added to the Carrel flask yielding a final pH of 6.8. The $Na_2C^{14}O_3$ solution was prepared by grinding 16.4 mg of $BaC^{14}O_3$ (200 μc) in 1.75 cc of CO_2-free double distilled water, and adding to this a solution of 23.7 mg of Na_2SO_4 dissolved in 1.75 cc of water. The mixture was agitated and the precipitated $BaSO_4$ was centrifuged down. After adding the $Na_2C^{14}O_3$ the flask was stoppered and shaken gently for 5 hours at 18° centigrade. The stages incubated were the two-cell or early cleavage stage (Shumway (14) stage 3), late blastulae (stage 9), yolk plug (stage 12), neurulae (stage 14) and tailbud larvae (stage 18).

The *Rana temporaria* were homogenized in lots of 300 in 3:1 alcohol-ether and lipid extractions were made from the homogenate. Cold 10 per cent TCA was employed for the extraction of acid-soluble materials. TCA was removed by ether before lyophilization of the fraction; the dry product was rewashed with ether and dried. After washing the acid-insoluble material with water and the lipid solvents, the dry residue was shaken with cold (2° centigrade) 1 N perchloric acid for 24 hours. This removes approximately 90 per cent of the RNA nucleotides (16), and the use of the Diesche diphenylamine test revealed no DNA present in this fraction. The pH of this solution was then adjusted to 2 and the nucleotides precipitated by adding 0.2 volumes of saturated $Hg(NO_3)_2$. The nucleotides were then ether-washed and dried. The DNA was solubilized by use of 5 per cent TCA at 90° for ten minutes (13). This fraction contained some RNA not removed by the perchloric acid. Ether was employed to remove the TCA before lyophilization and then again to wash

[1] 10 cc "A" solution and 14 cc of 0.025 M NaH_2PO_4. "A" solution consists of 3.4 g NaCl, 0.05 g KCl, 0.08 g $CaNO_3$, 0.1 g $MgSO_4$, and 500 cc water.

the dry product. The insoluble residue was washed with water, 3:1 and 1:1 alcohol-ether, ether, and dried. The proteins were then hydrolized in sealed tubes with 6 N HCl at 145° centigrade for 18 hours. The acid was removed by vacuum distillation and the amino acids were ether-washed and dried. The pigment residue was washed with water and the lipid solvents, and then dried.

Rana pipiens eggs were used for the study of regional $C^{14}O_2$ incorporation. Two lots of 300 normal early gastrulae (stage 10) and one of 300 neurulae (stage 14) were each suspended in 3–4 cc of a solution consisting of one part "A" solution[1] to one part of 0.05 M Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8) and transferred to a 15 cc Warburg flask. The isotope (200 μc $BaC^{14}O_3$) was placed in the side arm and perchloric acid was added to it after which the flask was immediately stoppered. The flasks were agitated for a five-hour period at 16.5° centigrade. The gastrulae advanced to stage 11 and the neurulae to stage 15. Development was arrested by washing with cold 10 per cent trichloracetic acid (TCA) and the embryos were cut and stored in this solution at 4° centigrade. The 600 gastrulae and 300 neurulae were dissected with glass needles according to Fig. 2. The gastrulae were divided into six parts; future ventral ectoderm, future neural ectoderm, dorsal lip, ventral lip, yolk, and ventral lip plus yolk as the yolk was not separated from some of the ventral lip quarters. The neural folds and underlying notochord and somites of late neurulae were dissected from 300 neurulae leaving the rest of the embryo as the other fraction. These morphological regions were then fractionated chemically. After homogenization in cold 10 per cent TCA, the acid-soluble and lipid fractions were removed according to Schneider (13); these fractions were discarded as these compounds could have diffused between regions of the individual embryos while they were being stored in cold 10 per cent TCA before being cut. The use of 5 per cent TCA at 90° centigrade for ten minutes solubilized the nucleic acids. This solution was ether-extracted to remove TCA, lyophilized and then further washed with ether. The residue left from the TCA fractionation consisted primarily of protein, but contained pigment. It was washed with water, 3:1 and 1:1 alcohol-ether, ether, and allowed to dry.

All samples were dried in an Abderhalden drier for several hours at the temperature of boiling chloroform. The dried samples were then combusted and collected as $BaC^{14}O_3$ in a 1 per cent $BaCl_2$-saturated $BaOH_2$ solution. The carbonate was washed four times with CO_2-free double distilled water and dried at 70°. The lipid $BaC^{14}O_3$ was counted on a thin window counter while all other samples were counted on a flow counter. Coincidence corrections were made for some of the high activity samples counted with the flow counter while other samples were diluted with ordinary barium carbonate before counting. $BaC^{14}O_3$ was plated on planchets by centrifuging down homogenized barium carbonate from an acetone suspension.

Due to small yields in the DNA fraction some of the tracer carbonate from similar developmental stages, but from different series of experiments, was pooled so as to allow a sufficient amount for counting. The nucleic acid $BaC^{14}O_3$ yields for the ventral and neural ectoderm, dorsal lip, and the neural folds - notochord - somites fractions were low and dilution with a known amount of nontracer barium carbonate in these three fractions was necessary to allow sufficient carbonate for plating.

[1] See note 1, p. 173.

RESULTS AND DISCUSSION

The various fractions from four complete *Rana temporaria* series were counted and the results, expressed in counts per minute/mg carbon, are illustrated in Table I and Fig. 1. Each point on the curves of Figure 1 is an

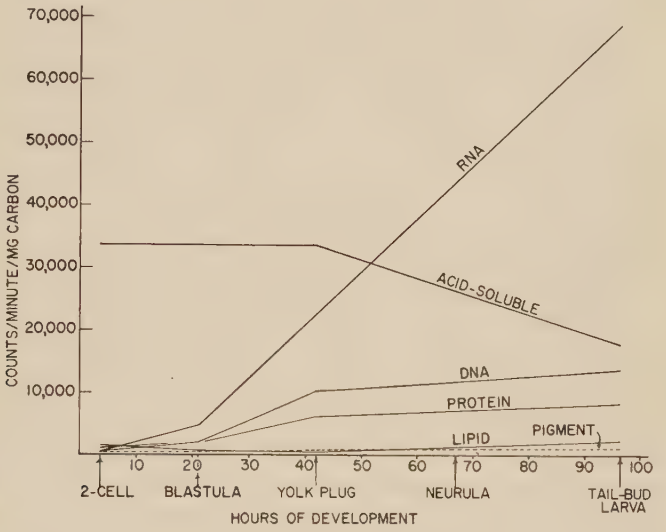

Fig. 1. Isotope activity of developing *Rana temporaria* embryos.

average of four determinations for that particular stage. The results clearly indicate a high acid-soluble and RNA activity and low lipid and pigment turnover. It should be noted from Table I that the observed level of tracer activity in the RNA, DNA, protein, and to a slight degree in the pigment fraction at the neurula stage is much lower than expected judging from the general slope of the curves. This observed drop is not thought to be a real drop and hence not plotted in Figure 1. Since this decrease in activity occurred in four different fractions it would appear that the pool of available carbon-14 was diminished at the neurula stage. Hultin and Wessel (10) have called attention to the greatly augmented rate of respiration after gastrulation in the sea urchin egg and the resultant dilution of $C^{14}O_2$ by respiratory carbon dioxide. In the frog embryo the same respiratory increase occurs (2) and a similar isotope dilution would be expected. Friedberg and Eakin (8) studying the uptake of labeled glycine found the total acid-insoluble activity of gastrulae to be three times that of the neurulae. This diminished activity was ascribed to the surface coat (9) of the amphibian egg which completely

covers the embryo at this stage. They had to cut neurulae in half in order to obtain adequate uptake of glycine C^{14}. It is believed the surface coat would also interfere with $C^{14}O_2$ and $NaHC^{14}O_3$ penetration into the neurulae and thus offer less available radio-carbon, thereby lowering the activity of several fractions.

TABLE I

Isotope activity of developing *Rana temporaria* embryos.
Results are expressed as the average counts per minute/mg carbon.

Stage	RNA	Acid Soluble	DNA	Protein	Lipid	Pigment
2-cell	382	33,661	453	1,088	1,336	399
Blastula	4,822		2,063	2,022	809	479
Yolk Plug	22,552	33,763	10,201	6,171	591	760
Neurula	24,722	26,696	6,937	5,419	1,093	754
Tailbud	69,852	17,914	13,886	8,281	2,302	1,046

The curve of tracer activity for the acid-soluble fraction is quite similar to those obtained by Kutsky (12) for *Rana pipiens* and Hultin and Wessel (11) for the sea urchin embryo where activity drops from a high level at the blastula and gastrula stages and thereafter one notes a shift of tracer from the acid-soluble to the acid-insoluble fractions. The lipid picture is again similar to that described by Kutsky. In both cases an initial fall in tracer up to the gastrula stage was followed by an increase thereafter. The level of fall in Kutsky's experiments was somewhat lower. According to the P^{32} data of Kutsky DNA isotope level is higher than that of RNA, but the $C^{14}O_2$ results show a higher RNA turnover.

One experiment was performed utilizing a centrifugal fractionation of homogenized radioactive neurulae. Centrifugation at 18,000 g for one half hour gave a supernatant and a residue fraction. The residue was well washed with water, alcohol-ether, ether, and dried while the supernatant was lyophilized. The supernatant level was 60,837 counts per minute/mg carbon, that of the residue was 3,533. Since almost all of the acid-soluble and RNA activity would be expected to lie in the supernatant, this high activity is not surprising. Most of the RNA is bound to the microsomes at this stage (4) and these particles would not sediment at the centrifugal forces used here. Hultin (11) has also shown that N^{15} from labeled glycine is incorporated *in vivo* into the microsome proteins at a more rapid rate than any other centrifugal fraction of livers of newly hatched chicks.

The regional incorporation of $C^{14}O_2$ into the protein-pigment and total

nucleic acid fractions of individual gastrulae and neurulae is illustrated in Figure 2. The results confirm the work of Friedberg and Eakin (8) and Eakin, Kutsky and Berg (6) in showing a real difference between the dorsal and ventral lips in terms of protein tracer uptake and extend their work by

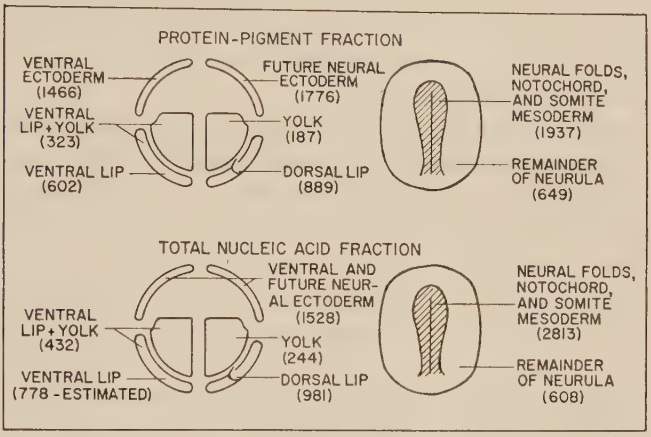

Fig. 2. Regional isotope incorporation into gastrulae and neurulae of *Rana pipiens*. Activity expressed as counts per minute/mg carbon.

demonstrating the same difference for the nucleic acids. Dorsal lip protein-pigment activity was 48 per cent greater than that of the ventral lip (based on equal amounts of carbon), while, from an indirect method, the dorsal lip nucleic acid activity appeared to be 26 per cent greater than that of the ventral lip. An indirect comparison was necessary since insufficient ventral lip material was dissected. The dorsal lip nucleic acid activity was 127 per cent greater than that of the ventral lip plus yolk nucleic acid, while the dorsal lip protein-pigment tracer level was 86 per cent above that of the ventral lip plus yolk protein-pigment fraction. Assuming the proportional differences in nucleic acid activity for ventral lip and ventral lip plus yolk to be comparable to those of the protein-pigment fraction of these parts, the dorsal lip nucleic acid activity is 26 per cent greater than that of the ventral lip.

Barth (3) has shown the respiration of the future neural ectoderm > future ventral ectoderm > dorsal lip > ventral lip > endoderm of amphibian gastrulae. In several of his experiments the dorsal lip showed more respiration than the ventral ectoderm, but the results listed above correlate directly with the isotope incorporation data of this study. For the protein-pigment fraction, the dorsal lip activity was only half that of the future neural ectoderm, and the ventral lip counts were 41 per cent that of the ventral ectoderm. The total nucleic acid incorporation of the dorsal lip was 64 per cent that

of the combined ventral and neural ectoderm. It is interesting that the higher level of dorsal activity also extends to the animal hemisphere of the egg. The future ventral and neural ectoderm are biologically equivalent at this stage, as transplantation experiments have shown that either one can respond to the dorsal lip contact stimulus and form neural tissue. However, the difference in isotope level between the ventral and dorsal lips may reflect the biological difference between them since only the dorsal lip material can induce the nervous system.

Regarding the neurulae, the carbon-14 level of the neural folds, notochord and somites was 198 per cent above the remainder of the embryo for the protein-pigment fraction and 362 per cent greater for the nucleic acids. The neural folds, notochord and somites had a protein-pigment activity only 9 per cent greater than the future neural ectoderm of the gastrulae, but the nucleic acid incorporation into these differentiating dorsal axial structures of the neurulae was 84 per cent greater than that of the gastrula ectoderm. This correlates with the results expressed in Figure 1 showing a gradually increasing level of isotope incorporation into the protein fraction and a very marked rise in the RNA fraction between the gastrula and neurula stages.

The endoderm has an extremely low level of tracer in either of the two fractions. This metabolic inertia may be ascribed to the yolk which is concentrated primarily in the endoderm region. Indeed it seems quite likely that the regional metabolic differences of the embryo are due to a differential distribution of yolk. The well known animal-vegetal pole yolk gradient could account for the higher level of isotope uptake in the animal hemisphere while the greater uptake of the future dorsal side of the embryo can be ascribed to fewer, and slightly smaller, yolk platelets on this side of the egg as compared to the ventral side. Shumway's (15) description of sections of blastulae and gastrulae of *Rana pipiens* illustrates this slight difference in yolk platelet distribution between the future dorsal and ventral sides of the embryo. That yolk is metabolically inert can readily be seen from cartesian diver respiration experiments upon tissue cultures (7). Oxygen consumption always increases after yolk platelet breakdown in the cells. Endoderm may have a very low turnover of C^{14}, but most of the egg protein is yolk protein, and total nucleic fractionations in this study have demonstrated that a good part of the nucleic acid of the egg is situated in the yolk endoderm. It is felt that despite the low level of tracer uptake in the yolk it is one of the most important factors in organization. Yolk constitutes a store of essential compounds and the differential regional localization of yolk platelets offers a reasonable explanation for some of the morphogenetic potencies and

metabolic differences which arise in the tissues. The importance being given to yolk is not due to its metabolic inertness but because it can, by virtue of containing the major share of the protein and nucleic acid of the embryo, offer metabolic and developmental possibilities which can be distributed regionally and utilized at different times in the development of the embryo. It is felt that the regional differences of isotope incorporation into gastrulae and neurulae are due to the spatial distribution of yolk platelets in the embryo and the temporal sequence of their decomposition. It appears that areas containing less yolk (animal hemisphere and dorsal side of the egg) have a more rapid metabolism; however, when the potential developmental energy of the platelets is utilized at the time of their breakdown, the rate of metabolism increases. The high isotope turnover of the neural folds, notochord and somite fraction is an example of this correlation between yolk platelet breakdown, metabolism, and differentiation. It is known that yolk platelet decomposition begins in the dorsal axial organs at about this stage of development (5).

It has been proposed by some investigators that certain materials might pass between tissues at the time of the contact stimulus of induction. The results of regional isotope incorporation do not provide any evidence for or against this scheme. The experiment of Abercrombie and Causey (1) does deal with this question. Donor neural tube tissue from a chick embryo marked with P^{32} was grafted to a host and a secondary neural groove was induced in the host by the transplant. However, the induced host tissue did not show any significant isotope activity as indicated by autoradiographs.

SUMMARY

1. A time course study of the isotope activity of various chemical fractions of *Rana temporaria* embryos incubated in the presence of $C^{14}O_2$ was carried out. The period of development studied was from fertilization to the tailbud stage. Individual gastrulae and neurulae of *Rana pipiens* were fixed in cold 10 per cent trichloracetic acid and regional carbon-14 incorporation determined in chemical fractions of the dissected parts.

2. The pigment, protein, DNA (desoxyribonucleic acid), and RNA (ribonucleic acid) fractions showed an increase of isotope incorporation during development. Lipid shows an initial decrease followed by an increase; the high acid-soluble uptake falls after the time of gastrulation.

3. Early gastrulae were incubated with $C^{14}O_2$, and the dissected parts showed the following order of isotope activity in the protein-pigment and

nucleic acid fractions: future neural ectoderm > future ventral ectoderm > dorsal lip > ventral lip > ventral lip plus yolk > yolk.

4. The tracer activity of dissected late neurulae reveals the major portion of the protein-pigment and nucleic acid activity to lie in the differentiating neural folds, notochord and somites rather than in the remainder of the embryo.

The author wishes to express his gratitude to Professor John Runnström for the hospitality of his laboratory and especially for his valuable suggestions concerning the initiation and conduct of this investigation. I am also indebted to Mr. Arlan Smith for his competent assistance during the latter part of this work.

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Experimental Cell Research, **6**, *181–194 (1954)*

CHEMICAL DETERMINATIONS OF THE EFFECT OF THE X AND Y CHROMOSOMES ON THE NUCLEIC ACID CONTENT OF THE LARVAL SALIVARY GLANDS OF *DROSOPHILA MELANOGASTER*[1]

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Received July 15, 1950

The problems of nucleo-cytoplasmic interrelations may be attacked by the study of the effects of variation in number of specific chromosomes on the nucleic acids of the cell. Measurements of the desoxypentose nucleic acids (DNA) should make it possible to estimate the content of the chromosomes themselves. Those of the pentosenucleic acids (PNA) give information with regard to the effects of chromosome function in nucleus and cytoplasm (1, 4). This paper presents chemical determinations of the quantities of these substances in larval salivary glands of *Drosophila melanogaster*, where individuals can be obtained varying in their number of X and Y chromosomes.

It may be useful to recall here some of the relationships of the X and Y chromosomes in *Drosophila melanogaster*. The male has one X chromosome, the female two, and both have three pairs of autosomes. The Y chromosome is the partner of the X in the male and is ordinarily not present in the female. It is larger than the X in the metaphase chromosomes and is composed chiefly of so-called heterochromatic regions. The genetic and cytological evidence indicates homology between the Y and the heterochromatic regions of the X chromosome. These regions, however, are duplicated several times in the Y chromosome and have possibly been differentiated in the course of the evolution of the species (review in 7). Because of these complications, the differences previously found (12) in nucleic acid content in male and female tissues provide a measure only of the effect of substituting a Y for an X chromosome. In order to analyze the separate effects of X and Y, it is necessary to produce females containing a Y chromosome and males which lack it. This can be accomplished by proper genetic crosses.

[1] Aided by an institutional grant from the American Cancer Society and by a research grant from the National Cancer Institute, of the National Institutes of Health, U.S. Public Health (C 1613) Service.

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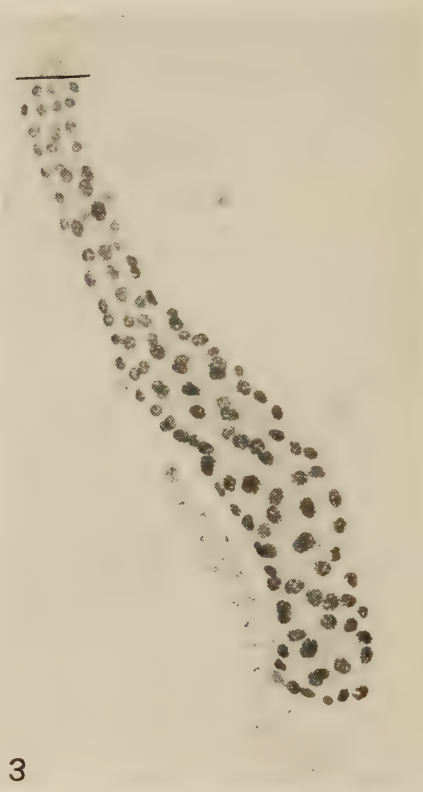
X
1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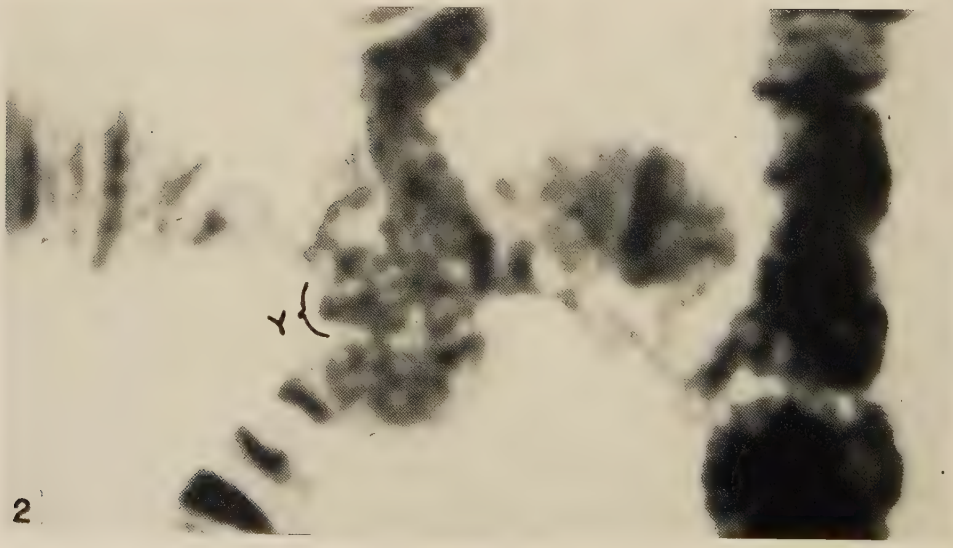
Y
2

There is already evidence to indicate an effect of the Y chromosome on the nucleic acids of the cell. Aside from cytologically observed effects on the other salivary gland chromosomes (15), microspectrophotometric measurements of the cytoplasm of oocytes showed a difference between the XX and XXY female; the presence of the Y chromosome increased the amount of substances absorbing at 2573 Å, probably PNA in the acetic acid fixed preparations used (5). This effect on the nucleic acids of the cytoplasm was considered remarkable, since otherwise there is little effect of deficiency or duplication of the Y chromosome on the normal *Drosophila* (7). The results were consistent with hypotheses derived from other data (4) relating heterochromatic regions to the metabolism of nucleic acids, and led to prominent mention of these chromosomal regions in theories relating nucleic acids to protein synthesis (1, 4).

To test the generality of these hypotheses, it seemed advisable to choose a material that differed from the oocyte in structure and function. The oocyte is a cell in which the nucleus is preparing for meiosis and the cytoplasm is being constituted to form precursors for the rapid mitoses which follow fertilization. Choice was indicated, therefore, of a cell in which mitosis will not occur and in which the synthetic process is secretion. The larval salivary gland of *Drosophila* fulfills these qualifications. In this organ, concomitant with the growth of the individual cells, there is an increase in nucleic acids in both cytoplasm (PNA) and nucleus (DNA and PNA) (4). The contrast with the oocyte is great, particularly during the phase of maximal growth; for the chromosomes of the oocyte remain small and contracted as opposed to the enormous size of the salivary gland chromosomes. The heterochromatic regions are different also. In the oocyte they are prominent, presumably like

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Fig. 1. Chromosome complement of a larval salivary gland nucleus from an XY individual of the Oregon R stock. X indicates the X chromosome, the heterochromatic end of which joins the chromocenter. The entire chromocentral region is enclosed in a square to indicate the region which is shown in higher magnification in the next figure. Acetic orcein smear. × 290.

Fig. 2. The chromocentral region. The Y chromosome comprises the small area to the right of the bracket. The Y corresponds to about 10 bands in the heterochromatic region of the X chromosome. × 4500.

Fig. 3. Whole mount of a larval salivary gland from a 5-hour (25°) prepupa, Oregon R, of XO genotype. In the glands used for chemical analysis, the imaginal disc cells are cut off with the duct, as indicated by the black line at the top of the gland. The fat body cells (seen on the left of the gland and containing small nuclei) are also carefully dissected away. Note the generally uniform size of the nuclei with the exception of smaller cells in the proximal portion of the gland. This gland was chosen to show the greatest variability seen in nuclear size. Some nuclei which appear to be exceptionally large are in reality two nuclei superposed due to the thickness of the gland. Feulgen. × 82.

We are indebted to Mr. David Hungerford for the photomicrographs.

glands from the four genotypes. In addition, the glands were examined microscopically in order to obtain a rough estimate of the maximum variability in nuclear size and degree of polyteny.

TABLE I

Numbers of Cells in the Different Types of Salivary Glands. Ranges of values in parentheses.

Sex	Genotype	Cell number	
		n	mean and standard error
♀	XX	20	148±2 (138—168)
	$X\overline{XY}$	19	134±2 (125—152)
♂	XO	21	145±2 (124—164)
	XY	18	148±3 (129—163)

Cell counts. The results of the cell counts are given in Table I. There is no significant difference between the means of the numbers of cells per gland in the case of glands from XX, XO and XY individuals, but glands from $X\overline{XY}$ flies gave a lower mean cell count. Counts were repeated on another culture of XO and $X\overline{XY}$ flies and the means of 22 and 24 glands checked the previous results closely: 144 for XO, and 134 for $X\overline{XY}$. An examination of the ranges in cell numbers shows that the $X\overline{XY}$ group lacks glands with cell numbers over 152. This phenomenon requires further study before the low cell number in this group can be considered valid. The cell number is fixed during very early embryonic development (14) and remains constant throughout the entire larval period, growth occurring by increase in cell size. Preliminary data indicate that the temperature at which mothers are raised may influence cell number in their daughters' salivary glands. In the present experiments, the temperature at which some of the mothers were grown probably varied between 20° and 25°. Counts made previously (12) on salivary glands from the giant apricot stock with the mothers grown at 25° showed no significant difference between the means of the numbers of cells per gland in glands from males and females, giants and non-giants. In view of the uncertainties regarding the control of cell numbers, it seemed advisable to present the results on both a per gland and a per cell basis, relying principally on the per gland basis for the comparisons.

Nuclear variability. The larval salivary gland of the five-hour prepupa was chosen for these experiments since at this stage of development the variability of the nuclei is least, growth has reached a maximum, and pupal histolysis has not yet set in. Here the progressive maturation (distal to proximal) of the cells is complete (2). Furthermore, at the five-hour prepupal stage, there is no obvious histolysis in the Oregon R stock of *D. melanogaster.* This conclusion is based on earlier studies (13) of the alanylglycine peptidase content of glands at different times after formation of the prepupa. A peak in enzyme activity is reached at the six-hour stage after which glands showing microscopically visible signs of disintegration may give lower peptidase values.

Undoubtedly two types of cells exist in the larval salivary gland (Fig. 3). According to our own unpublished observations on *D. melanogaster* and also those on *D. robusta* (8), there are small cells grouped in the proximal part of the gland adjacent to the imaginal disc cells (forerunners of the adult gland), which differ from those in the rest of the gland in that they do not contain Hotchkiss positive material with which the rest of the secretory cells are loaded. These are cells (designated duct cells here) in which the nuclei are small and the chromosomes thin stranded. The number of these nuclei in the proximal part of the gland varies between 10 and 26 with a mean of about 21. The remaining 125 nuclei in the secretory cells are chiefly of one type, although some smaller nuclei are present and occasionally there is one with very large chromosomes, probably tetraploid. Therefore it can be concluded that in the 36 glands used for one extract, approximately 4 500 cells are of the large secretory type, and 750 of the type that forms the proximal ductlike part of the gland. Even with 14 per cent of the cells belonging to a second type, this tissue is more uniform than most used for biochemical studies. The important point to determine for this work, however, is whether the glands with different numbers of X and Y chromosomes vary with respect to numbers of these two kinds of cells.

Accordingly, cytological observations were made on Feulgen whole mounts, twelve for each of the four genotypes. The number of the non-secretory duct cells, with low grade of polyteny, was counted in all four genotypes. The results showed as great a variability between the members of a pair of glands as that between the various genotypes. Thus, at least as regards the number of duct cells, no significant differences between the four groups appear to exist.

In addition to the counts of duct cells, attention was also directed to the possibility of a cytological distinction between the genotypes, on the basis

of differences in the overall grade of polyteny. Judging by the thickness of the chromosomes large changes in polyteny (as between duct cell and secretory cell) could be distinguished easily; however, smaller differences were far more difficult to evaluate. Nevertheless an attempt was made to classify the forty-eight slides into two groups, the genotype being unknown to the observer. One group contained a high frequency of cells with pale diffuse nuclei in which the chromosomes were slender; in the other, the majority of the nuclei contained thicker chromosomes. These observations were made at relatively low magnification (500 ×, objective 44 ×), and were quite rough. Earlier cytological study (15) had shown differences in the "plumpness" and in the characteristics of the ends of the chromosomes in the various Y chromosome types. Here the difference in the ends could be seen but was not taken into consideration in the classification into two groups. When the four genotypes were compared, it was found that each contributed equally to both groups, within statistical limits. Hence, with this admittedly crude method of estimating relative polyteny, no distinction between genotypes could be seen that would have resulted from a difference in cell differentiation expressed in terms of growth of the chromosomes. Therefore valid comparison of the types of glands could be made.

Chemical determinations

Distribution of phosphorus. Before considering results of analyses for DNA and PNA in the hot perchloric acid (PCA) extracts, it is advisable to study the distribution of phosphorus (Table II) between the alcohol-ether, cold PCA and hot PCA extracts and the residue. The low percentage of phosphorus in the residues indicates complete extraction of nucleic acids. The distribution of phosphorus between the other three fractions is similar to that found previously (12) in the case of the same stage salivary glands from the giant

Table II

Distribution of Phosphorus in Extracts of Salivary Glands.

Sex	Genotype	Alc. Ether	Cold PCA	Hot PCA	Residue	Total P
		%	%	%	%	mμg/gland
♀	XX	32.4	18.3	48.1	1.1	99.8
	XXY	34.5	19.7	44.8	0.9	105.3
♂	XO	34.0	16.5	48.7	0.7	85.9
	XY	34.4	15.5	49.2	0.9	83.0

apricot stock of *D. melanogaster*. There is no difference in distribution between the four types of glands; no effect of either the X or Y chromosome is evident. Nevertheless, the total phosphorus (given in mμg per gland in the column at the extreme right of Table II) is higher in glands from females than in those from males, giving a female to male ratio similar to that found previously (12). The presence of a second X chromosome, therefore, results in an approximately equivalent increase in the phosphorus-containing constituents of all fractions. No consistent action of the Y chromosome is seen.

TABLE III

Analysis of Cold PCA Extracts.
Means and standard errors of the mean are given as mμg/gland calculated as phosphorus.

Sex	Genotype	P (orc)	P (uv)	P (found)
♀	XX	6.9±0.4	6.9±0.6	21.0±2.3
	XXY	7.1±0.4	7.2±0.4	20.8±1.4
♂	XO	5.2±0.3	4.7±0.4	14.4±1.1
	XY	5.2±0.4	4.4±0.3	13.0±1.1

Analysis of the cold PCA extract. A confirmation of the effect of the X chromosome is seen in the results of the analysis of the cold PCA extract (Table III). The values are given in mμg per gland and the results from the orcinol reaction and ultraviolet absorption curves are calculated in terms of phosphorus equivalents (based on a nucleotide with one atom of phosphorus) for comparison with the total phosphorus. As previously found in other stocks (12), the molar ratio of pentose to purine plus pyrimidine to phosphorus is 1 : 1 : 3. These values could represent a nucleotide such as ATP or a mixture of other acid-soluble phosphorus compounds including nucleosides and nucleotides. One chromatographic analysis carried out in this laboratory for us by Dr. Levenbook and Miss Travaglini indicated the presence of uracil, hypoxanthine and possibly adenine in a formic acid hydrolysate of pooled cold PCA extracts. For the purpose of this paper, however, the main points are that the molar ratios are the same in the four types of glands; that the female to male ratio seen in the total phosphorus is also present in this extract; and that no effect of the Y chromosome is apparent.

Analysis of hot PCA extracts — DNA. Having established that the extraction procedure gave equivalent results in the first two extracts, we can proceed to the analysis of the nucleic acids extracted with hot PCA. Table IV gives the DNA values found both on a per gland and on a per cell basis. Calculated either way, the DNA content of the females is higher than that

of the males, but only just significantly so (P =0.01–0.02) on a per gland basis when both X and Y are added as in the comparison XXY and XO; when X alone is added as in XO to XX and XY to XXY, the difference is of dubious significance (P=0.1). This 5 mμg per gland difference corresponds to a density reading of only about 0.004 density units which, as mentioned in the section on methods, is below the value necessary for a significant measurement. Obviously the 2 mμg per gland value for the Y chromosome has no

TABLE IV

The Desoxypentosenucleic Acid (DNA) Content of the Different Types of Salivary Glands. Means and standard errors of the means. Ranges of values in parentheses.

Sex	Genotype	DNA		
		per gland		per cell
		n	mμg	μ μg
♀	XX	5	36±2 (32—42)	245±13
	XXY	5	38±1 (35—39)	281± 6
♂	XO	8	31±2 (22—43)	217±12
	XY	9	33±2 (23—38)	221±14

significance. Other methods must be found if actual DNA quantities per chromosome are to be determined. Use of longer absorption cells would increase the density readings but a better solution of the problem may rest on isolation of the nuclei in a countable condition and separation of the two classes of nuclei. Experiments under way indicate that this may be feasible.

PNA. Finally, the results which pertain directly to the relation of the chromosome complement to PNA are given in Table V. The PNA content of the four types of glands is expressed on both a per cell and a per gland basis. On either basis, the PNA values of the females are significantly higher than those of the males, the female to male ratio being 1.2 as found previously for the giant apricot Oregon R stock (12). On a per gland basis, there is clearly no significant difference between XX and XXY or XO and XY; therefore, neither the addition nor the deficiency of the Y chromosome influenced the content of PNA to an extent measurable by the present methods. On a per cell basis, the lower cell number in the XXY group (Table I) results in a barely measurable increase (significant at the 5 per cent level) in the PNA

per cell compared to the XX group. Considering the above-mentioned uncertainties in our knowledge of factors affecting the cell number in the embryo, this increase cannot be given serious consideration. In addition, there is no significant difference in the PNA per cell in the XO and XY group. So it can be concluded that the Y chromosome in this material has no measurable effect on the PNA content.

TABLE V

The Pentose Nucleic Acid (PNA) Content of the Different Types of Salivary Glands. Means and standard errors of the means. Ranges of values in parentheses.

Sex	Genotype	PNA		
			per gland	per cell
		n	mμg	$\mu\ \mu g \times 10^{-1}$
♀	XX	5	455±14 (408—491)	308±11
	$\overline{XXY}$	5	449±7 (435—465)	334± 7
♂	XO	8	376±7 (332—436)	260± 7
	XY	9	365±6 (343—412)	246± 6

In addition to the analyses on salivary glands from the Oregon R stock, a few experiments were carried out with the giant apricot Oregon R stock, comparing XY with XO in glands from both giants and non-giants. The results were similar to those obtained with the Oregon R stock; no clear-cut effect of the Y chromosome on the nucleic acid content could be measured.

DISCUSSION

The difference between the effect of the Y chromosome on the PNA of the oocyte and of the salivary gland has some theoretical importance, if correct. It indicates that for so general a constituent of cells as the pentose nucleic acids, substances not ordinarily considered as products of cell specialization, the functioning of the chromosomes in different tissues may vary (cf. 17). Moreover, following this line of thought further, it would appear that the heterochromatic regions, of which the Y chromosome is a prime example, also differ in their functioning in different tissues. Hence, the hypothesis (4) that they serve to control the supply of "non-specific" precursor substances for cytoplasmic protein synthesis would become com-

plex, involving different capacities for the utilization of the uniformly produced precursors in the different tissues. These considerations have sufficient general relevance to exact stringent requirements from the data.

The first requirement is that the methods used for the two sets of measurements be comparable. Actually, the microspectrophotometric data (5) have been called into question by Callan (3; see also 16) on the basis of determinations of nucleic acids in *Drosophila* eggs. Since he used a now outmoded chemical method (furfural determination), a discussion of the nucleic acids and their precursors in the egg may be deferred. The problem is now under investigation in this laboratory (9) and it may be stated that analytical data are at hand indicating a far more complex situation than was apparent with the earlier methods. These analyses apparently confirm the inference from the absorption spectra (5) that the major proportion of the absorption of the egg cytoplasm at 2573 Å is in fact contributed by the pentose nucleic acids. Pending the results of these further investigations, the early results are best interpreted to mean an actual difference in the content of pentose nucleic acids in the oocytes of the XX and XX$\bar{Y}$ females, in contrast to the results here reported on the salivary glands.

Still further questions arise with respect to the comparison of the stages of development of the two types of cell. The oocyte measurements were made on cells from the egg chambers of the young ovary during the growth period. But the measurements of the salivary gland cells were made on glands from five-hour prepupae at a stage when larval development is completed. It is conceivable that differences in the PNA per cell could be detected only at early stages, that, for example, the quantity of heterochromatin only affects the rate of synthesis. This possibility cannot be excluded at present and even has some plausibility. Microspectrophotometric studies of the salivary gland at early stages, as well as comparisons of the mature unfertilized egg in the various Y chromosome types, should be useful in answering these questions.

With the effect of the Y on the PNA content of the salivary gland cells negligible, the difference in PNA between the male and female groups can now definitely be ascribed to the relation between the X and the autosomes. This difference, as has previously been shown (12), accompanies other characteristics of the cell types: total nitrogen, volume, alanylglycine peptidase content, total phosphorus content. It obviously offers material for further study of the relation of nucleic acids to other syntheses of the cell, particularly in view of the fact that there is a change in the ratio of nucleic acids to proteins during growth of the female gland cell (4, p. 125). For the present, discussion may be restricted to the relation of the PNA to the DNA content of the cell.

The chief question is whether the different PNA to DNA ratio of the male and the female is the direct consequence of the lack of one X chromosome, or whether the action is indirect, through an effect on the degree of polyteny which the salivary gland chromosomes attain in the different sexes. The cytological observations, it will be remembered, showed no distinction between the average polyteny of the different genotypes. This conclusion is born out by the chemical measurements, within the limits of their accuracy. The DNA value per nucleus is an average to which the respective chromosome arms contribute according to their lengths. Disregarding the small fourth chromosome as negligible in this discussion, the average DNA per haploid chromosome arm is one-tenth the total in the nucleus. According to the chromosome maps of Bridges, the average number of bands per chromosome arm is 1005, not far from the 1024 in the X chromosome. Considering the female value first, the XX female contains 245 μμg per nucleus, which gives a value of 24.5 μg per haploid strand. This is not far from the value obtained from the XO data, in which 217/9 gives the value per haploid strand of 24.1. On this basis, the degree of polyteny, as measured by the DNA per strand, is the same in the XX female and the XO male. But the difference between these two values, as previously discussed, gives an estimate of the DNA content of the X chromosome—actually, 28 μμg. This should be, from the chromosome map, close to the value of the average haploid strand, if no differences in polyteny exist between the two genotypes. With the reservations necessary from the limits of accuracy of the DNA determinations, the agreement is surprisingly good. Altogether, the evidence indicates that no great differences in the average degree of polyteny are encountered in the material of this paper.

The discussion of the preceding paragraphs leaves the change in PNA content with the addition of the X chromosome as an effect of the X itself. The problems raised by this conclusion are familiar ones in the genetic literature (11). Especially in comparisons of the expression of sex-linked genes, importance has been given to the fact that the male lacks a set of X chromosome genes, yet in most sex-linked mutants there is little sexual dimorphism. There would seem to be a compensation in the requirements for X chromosome genes such that when a whole X is lacking a viable male genotype results, balanced in the proportions of the different types of genes. There are, however, some X chromosome mutants in which the expression is different for two genes, as opposed to one. It would follow that those genes in the X chromosome concerned with PNA synthesis belong to the latter category, for which dosage compensation is not complete.

Finally the X chromosome genes having to do with PNA synthesis deserve

mention in relation to the heterochromatic regions. Since the addition of the Y chromosome produces no notable change (except in the dubious $X\overline{X}\overline{Y}$ value per cell), the homologous heterochromatin of the X chromosome cannot be held responsible for the additional PNA of the female. Other X chromosome genes must be involved, but it would be premature to identify these with the intercalary heterochromatic regions. From the point of view of genic balance, it would seem most reasonable that many diverse chromosome regions, "euchromatic" as well as heterochromatic, are involved in nucleic acid synthesis. The variety of metabolic pathways traced out by the isotopic studies is surely consistent with such a point of view (6).

SUMMARY

Chemical determinations of desoxypentose (DNA) and pentose (PNA) nucleic acids were carried out on mature larval salivary glands from *Drosophila melanogaster* which were either of normal constitution (XX or XY) or contained an extra ($X\overline{X}\overline{Y}$) or lacked a Y chromosome (XO). Both DNA and PNA contents were found to be higher in the females than the males, presumably due to the additional X chromosome of the female. On a per gland basis, neither the addition nor the deficiency of a Y chromosome influenced the content of either PNA or DNA to an extent measurable by the present methods. The results are discussed with reference to the function of the heterochromatic regions, and the relation of the DNA content of the nucleus to the PNA content of the cell.

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Experimental Cell Research, **6**, *195–201 (1954)*

THE SUBMICROSCOPIC STRUCTURE OF THE BALBIANI-RING

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Received August 4, 1953

Since the work of Pease and Baker (9) several attempts have been made to obtain direct electron optical proof of the polytene theory of giant chromosome organization. However, the evidence presented either in favour (9, 10, 11) or against (3, 8) this theory cannot be considered as convincing since the techniques used involved inadequate procedures, such as acetic acid treatment for squashing, dissolving the embedding medium after sectioning, and in most cases drying of the preparation. Only very recently techniques have been developed in electron microscopy which are satisfactory from the cytological point of view: fixation in buffered OsO_4-solution according to Palade (7), embedding in methacrylate polymer according to Newman, Borysko and Swerdlow (6), and ultra-thin sectioning and investigation of the sections without dissolving the embedding medium as first proposed by Hillier and Gettner (4). Borysko (3) has used this method, but replaced the embedding medium by collodion.

Freshly dissected salivary glands of larvae of *Chironomus tentans* Fabr., and one other undetermined *Chironomus* species, were placed over OsO_4-vapour for 4 minutes and then fixed for 2 hours in 1 per cent OsO_4 in distilled water, buffered at pH 7.2 with veronal. After embedding in methacrylate the glands were sectioned with a Spencer No. 820 rotary microtome adjusted to 0.1 μ, using specially sharpened steel blades.[1] After stretching on 50°C warm distilled water, the sections were transferred to collodion-coated specimen holders. The instrument used was an RCA EMU type electron microscope.

The advantages of the new combined technique are obvious from Fig. 1: The general appearance and especially the banding pattern of the salivary gland chromosomes as seen in the electron microscope correspond completely to the pictures obtained by means of the UV or ordinary light microscope. Remarkably enough, the thicker bands are not compound, in the sense that they would consist of subunits of uniform thickness corresponding to the

[1] F. Sjöstrand, Personal communication.

thinnest bands to be seen. At least, no such subdivision is detectable. Some double or triple bands may occur, but they generally are known already from light microscopical studies. On the other hand, the thinnest bands, ranging from 0.1 μ down to about 0.05 μ (Figs. 2 and 3), certainly are not revealed light-optically, even in optimally stretched chromosome parts in squash preparations. Their number, however, does not seem to be very large, so that the number of bands in cytological maps of giant chromosomes would not have to be multiplied by a factor much larger than 2. The total would still not exceed gene numbers as estimated from genetic evidence.

The main interest in this study was directed to the structure of certain regions of the 4th chromosome of *C. tentans* which in the salivary glands constantly appear modified as "Balbiani-rings". From earlier observations (2) it was known that these structural modifications are an expression of high physiological activity of the regions involved. In the light microscope, Balbiani-rings have a certain resemblance to nucleoli but differ from them in several aspects, mainly their diffuse though regular contour. The adjacent chromosome sections split up progressively until the thinnest strands disappear within the diffuse, predominantly peripheral, parts of the ring (1). On the basis of the polytene theory it has been assumed (2) that the individual elements ("chromonemata") of the giant chromosome completely give up their lateral union for a short part of their length at these points, each one forming a loop before uniting again with other elements to resume its helical course within the chromosome-"cable". This interpretation has now been checked electron-optically as shown in Figs. 5–8.

The photograph in Fig. 5 has been obtained from an oblique section through the big Balbiani-ring of the 4th salivary chromosome of *Chironomus tentans*. The majority of the branchings of the adjacent chromosome regions running into the ring have been cut transversely or obliquely—if hit longitudinally they clearly show their banding (lower right of Fig. 5). Between these, the light-optically diffuse parts of the ring appear as a mass of extremely spongy, uniform elements which at the periphery clearly appear curved, at some points even show complete loops. It is suggested that these are the

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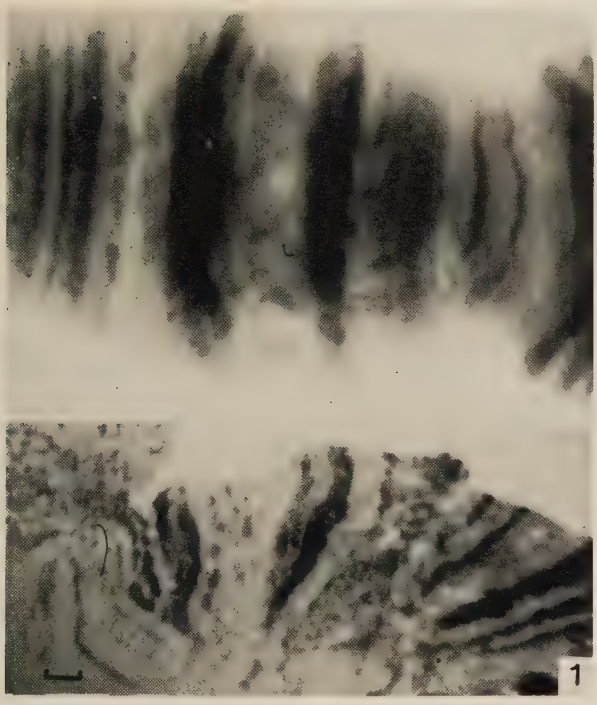
Fig. 1. Regions 16 and 17 of the 2nd salivary chromosome of *Chironomus tentans* in the UV (upper photograph) and in the electron microscope, both pictures at the same magnification, 4,700 ×. The UV photograph is from an unstained, acetic acid fixed squash preparation mounted in glycerol, the electron photograph from an ultra-thin section (see text for technique.)
Fig. 2. Region 17 B of the 2nd chromosome, in the UV and in the electron microscope, 10,000 ×.
Fig. 3. Right end of the 2nd chromosome showing the "telomere", a heterochromatic band of only about 0.05 μ thickness, 30,000 ×.
Fig. 4. Partial section of one nucleolus together with its organizer, 12,000 ×.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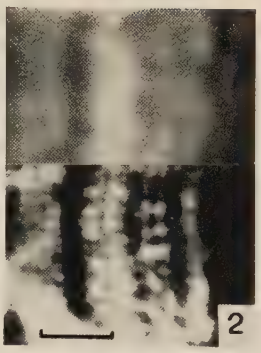
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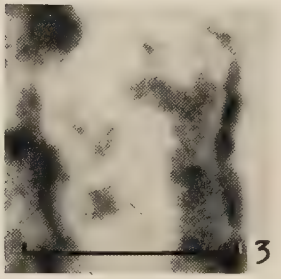
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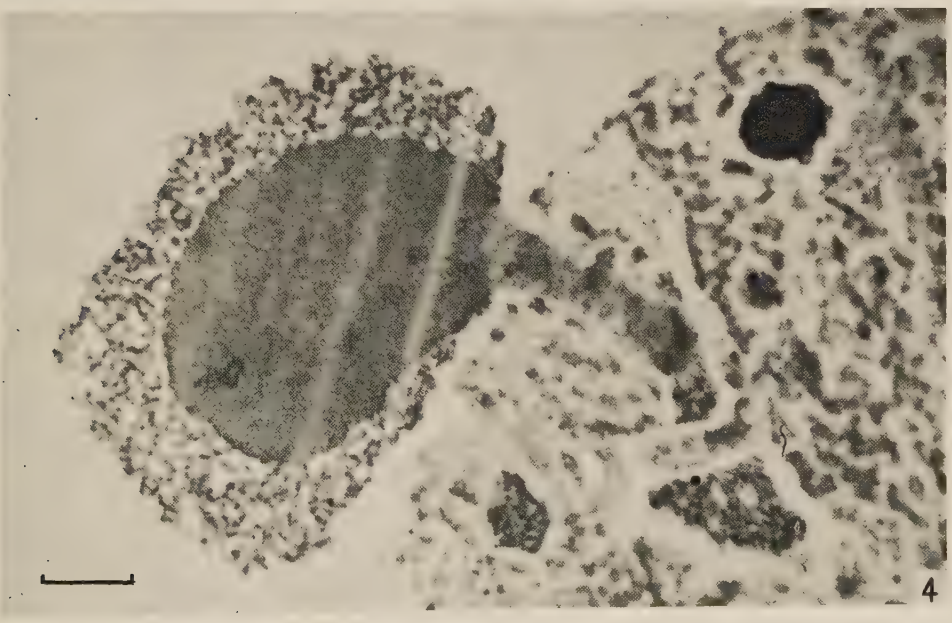
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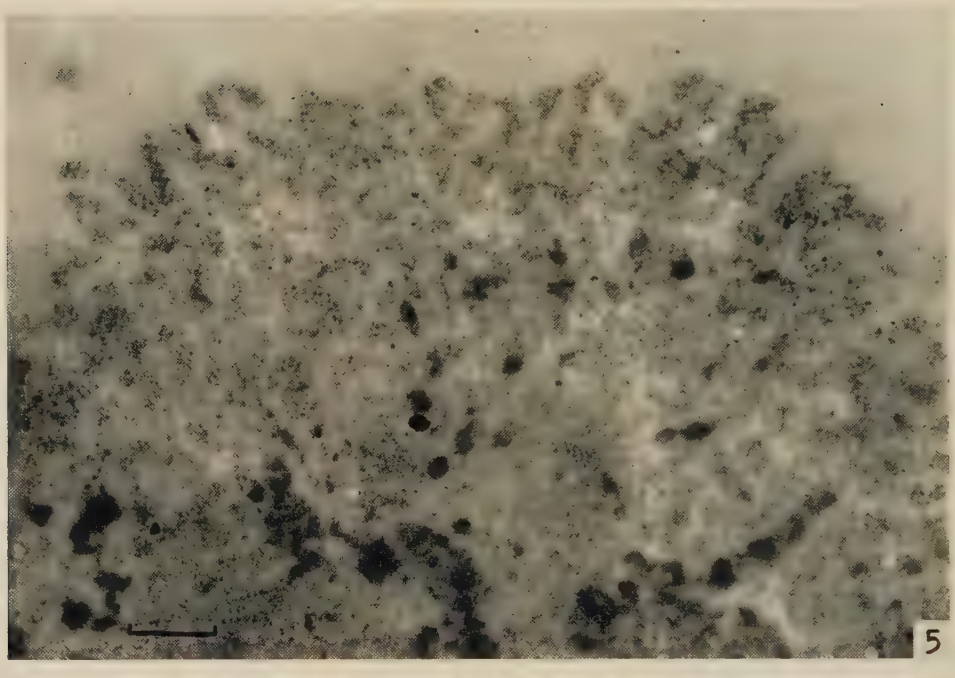
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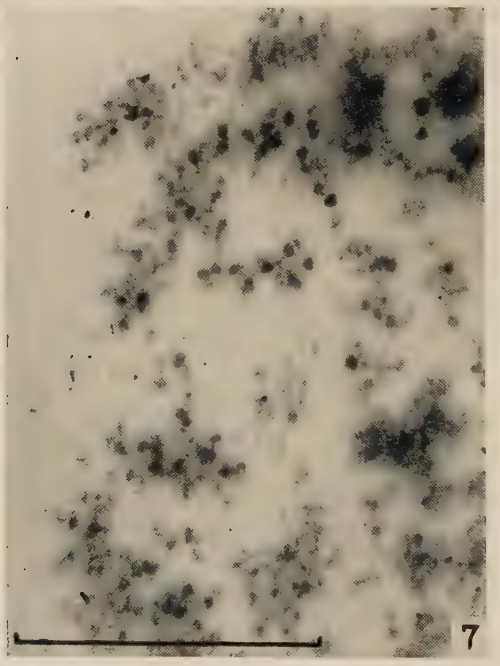
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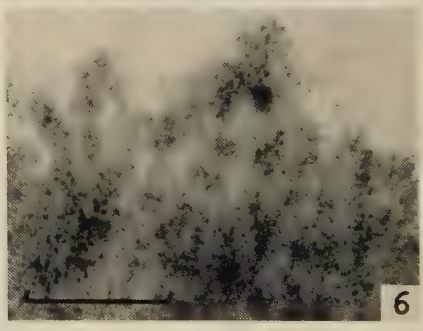
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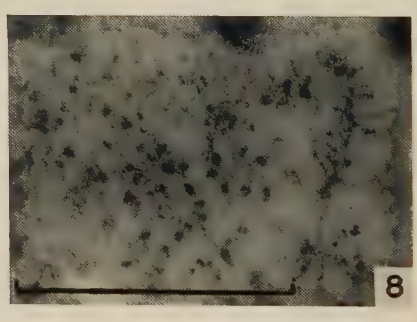
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ultimate longitudinal units of the giant chromosome, though obviously in a special structural state: certainly they are not just smooth fibrils. In high-resolution photographs (Figs. 7 and 8) each individual element resolves itself into a loose system of irregularly zig-zagging threads giving the impression of a "lamp-brush" (not to be confused with the "lamp-brush" chromosomes of amphibian oocytes). The existence of a continuous axial fibre can only be guessed, whereas numerous branchings of about 10 mμ thickness are clearly visible. They bear characteristic spherical bodies of a maximum diameter of about 30 mμ, apparently attached to their ends. These globules never appear at other points of the chromosomes. The whole "chromonema" in the condition described has an average diameter of about 0.2 μ, twice as much as the finest still compact, hence light-optically discernible, branchings of the chromosome, cross sections of which appear as dark spots a few micra distant from the periphery of the ring. As judged from this distance the length of the "lamp-brush" segments of the chromonemata may reach 4 μ. Continuous pieces of about 2 μ are clearly seen at some points of the section in Fig. 5.

These observations may be interpreted as follows: As has already been inferred from light-optical studies, the "active" segments of the chromonemata within the Balbiani-ring are in an extremely extended state, possibly more than 5 times their normal length, because the development of Balbiani-rings can be traced back to single bands or interband regions of no more than 0.5 to 1 μ thickness under normal circumstances. Since the giant chromosomes themselves have up to 10 times the length of the pachytene chromosomes, this means that the "lamp-brushes" are 50 times longer than the corresponding segments at pachytene, and yet, functionally, they may constitute nothing but one single gene locus. In addition, the "lamp-brushes" show a considerable degree of lateral "puffing" as judged from the fact that the immediately neighbouring branchings of the chromosome are only half as thick and still probably contain 2 or more of the ultimate elements. Unfortunately, points of transition between the finest branchings of normal structure and the "lamp-brushes" could not be demonstrated as yet. The blown-up condition of each individual "Balbiani-locus" has to be considered as the main cause

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Fig. 5. Part of an oblique section through the big Balbiani-ring of the 4th chromosome of *C. tentans*, 12,000 ×.
Fig. 6. Peripheral part of the Balbiani-ring of another undetermined *Chironomus* species, 20,000 ×.
Fig. 7. High-resolution photograph of the periphery of a Balbiani-ring, 42,000 ×.
Fig. 8. Part of Fig. 5 at a higher magnification, showing the "lamp-brush" structures referred to in the text, 38,000 ×.

of "puffing" of the whole Balbiani-ring, as discussed by Mechelke (5), and certainly has some causal relationship to the formation of the characteristic globules mentioned above. Sufficiently thin sections of normal bands show what seem to be closely packed thread-like elements of the same dimensions as those composing the "lamp-brushes". Failure to demonstrate the individual "chromonemata" within the normally built regions of giant chromosomes may then be explained as a consequence both of their complicated structure and close union.

The question of the degree of polyteny of giant chromosomes has often been discussed. The number of "lamp-brushes" within the total area of the particular section, part of which is shown in Fig. 5, may roughly be estimated to lie between 1,000 and 2,000. Since the "lamp-brush" segments are rather short (2–4 μ) as compared to the diameter of the Balbiani-ring (about 20 μ), obviously only part of them is included in the section. The total may amount to 10 times as much, which means that the paired salivary chromosome of a full-grown *Chironomus* larva may contain more than 10,000 longitudinal elements. This value is in agreement with earlier estimates based on nuclear measurements (2), if the number of chromonemata within the original telophase chromosome, before it becomes polytene, is assumed to be very small.

Some accidental observations on the salivary gland nucleoli of *Chironomus tentans* may be added here. Light-microscopically, the nucleolar substance often, but not always, is seen to consist of two zones, a central clear one and a peripheral, granular one. Islands of the granular substance may be included in the clear zone. The nucleolar organizers are diffusely staining (acetocarmine) interband regions of about 0.5 to 1 μ thickness. In the electron microscope, after osmium "staining", the central zone of the nucleoli appears highly absorbing and finely granular, while the peripheral substance is seen to consist of loosely packed, short, rod-like particles (Fig. 4). Points of transitional structure may be found. With its central, denser portion the nucleoli are attached to the organizers which have a slightly lesser absorption than the nucleolus itself.

The preliminary observations reported here are in full accordance with the polytene theory in general, and with the concepts of Balbiani-ring structure proposed earlier (2, 5). OsO_4-fixation may not be able to preserve or demonstrate all details of living structure, and in the interpretation this has to be kept in mind. However, no better fixative is known at present, and, by generally accepted cytological standards, the structures it does preserve are in excellent condition.

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ANTIGENS IN SEA URCHIN HYBRID EMBRYOS

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Received August 10, 1953

Students of embryology have for many years attempted to clarify the role of the cell nucleus in early embryonic development. There have been various experimental approaches. One approach of some interest is that of cross-fertilization, and there are numerous reports on the development of hybrid embryos (e.g. 11, 12, 16, 17, 19, 20).

Serological methods have been used in the study of embryonic development (6–9, 21, 27–31), and in detecting species specific substances in adult forms (see Boyden, 5, for a review). The purpose of the present investigation is to extend the study of hybrids to the biochemical level; specifically to determine the serological properties of the eggs and early developmental stages of three species of sea urchin and of their hybrids. Although much remains to be done on this problem, we feel justified in reporting this work now, as it may be some time before we will again be able to obtain material from these species.

MATERIALS AND METHODS

Arbacia lixula, *Paracentrotus lividus*, and *Psammechinus microtuberculatus*, collected in the Bay of Naples, were used. In addition to the normal embryos of each of these species, hybrid embryos of each of the crosses shown in Table I were cultured.

The fertilized eggs were washed carefully in filtered sea water within 1 to 2 hours after fertilization. In the case of the *Paracentrotus* ♀ × *Arbacia* ♂ cross, where high concentrations of sperm were used, three washings were performed at this time. Mass cultures were maintained according to the method of Lindahl (18). The cultures were washed again at approximately 24 hours, and, in the longer cultures, again at 48 hours. The embryos were finally centrifuged into a tightly packed layer and the supernatant sea water decanted. The sediment was lyophilized and stored at —20°C. Embryos were quick-frozen at the following times: *Paracentrotus* ♀ × *Psammechinus* ♂ at 24 hours (blastulae after formation of primary mesenchyme,

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see Hörstadius ((13), p. 134, Fig. 1), *Psammechinus* ♀ × *Paracentrotus* ♂ at 30 hours, *Paracentrotus* ♀ × *Arbacia* ♂ at 35 hours, and all hybrids and parental species at 48 hours (early plutei), and *Psammechinus* ♀ × *Paracentrotus* ♂ at 93 hours.

TABLE I

Hybrids used and methods employed for obtaining over 90 per cent cross fertilization.

Hybrid	Treatment of gametes to facilitate crossfertilization
Psammechinus ♀ × *Paracentrotus* ♂	Eggs fertilized in 0.1 per cent glycine in sea water.
Paracentrotus ♀ × *Psammechinus* ♂	Eggs treated with 0.05 per cent non-crystalline trypsin for 10–15 mins. (see Hultin, 14), washed, fertilized in 0.1 per cent glycine in sea water.
Paracentrotus ♀ × *Arbacia* ♂	Eggs fertilized in 0.1 per cent glycine and *Arbacia* egg water,[1] fertilized with high concentration of sperm.

The serological investigations were carried out in Stockholm. The lyophilized materials of 48 hour embryos of each of the species and of one of the crosses (*Psammechinus* ♀ × *Paracentrotus* ♂) were each suspended in 0.14 M NaCl buffered to pH 7.4 with phosphate buffer, and injected into rabbits. Three rabbits were used for each kind of material. For the precipitation tests, antigen solutions of the 48 hour embryos of each of the species and each of the hybrids were made up as follows: 100 mg of the lyophilized material was suspended in 10 ml 0.14 M NaCl buffered to pH 7.4 with phosphate buffer, and vigorously stirred by means of an air-driven glass stirrer for 2 hours in the cold. The preparations were then centrifuged at 10,000 rpm for 2 hours in the cold. The supernatant was dialyzed against 0.14 M NaCl for 24 hours in the cold. The sediment was washed several times with 0.14 M NaCl and then extracted with 2 M NaCl buffered to pH 7.4 for 2 hours, and this extract centrifuged and the supernatant used undiluted without further treatment. The dialysate of the 0.14 M NaCl extract was centrifuged in the ultracentrifuge at 65,000 × g for 2 hours. A nitrogen determination of the supernatant was made and the solution diluted so that the final solution contained 0.1 mg nitrogen per cc. Similar extractions were made of lyophilized *Paracentrotus* ♀ × *Psammechinus* ♂ (24 hours), *Psammechinus* ♀ × *Paracentrotus* ♂ (30 hours), *Paracentrotus* ♀ × *Arbacia* ♂ (35 hours), and *Psammechinus* ♀ × *Paracentrotus* ♂ (93 hours).

The authors felt that the washings of the cultures were sufficient to remove contaminating sperm which might affect the results. However, in order to check on the possibility that contamination by sperm might give misleading results, two types of control experiments were carried out. The first type of control consisted

[1] To obtain the stock solution of egg water, washed *Arbacia* eggs were allowed to stand in about 10–15 times their volume of sea water for 6 hours with frequent gentle stirring. At the end of this time the eggs were allowed to settle, and the supernatant decanted and filtered. This stock solution was kept in the cold until needed. For an experiment it was diluted so that the final concentration in the fertilization medium was 1/6 of the original concentration.

of mixing eggs and sperm of different species, allowing the mixture to stand for an hour, washing this mixture once, and then lyophilizing and extracting as described above. As a further check, 1 g of lyophilized egg material of one species was experimentally contaminated with 0.1 g lyophilized sperm from another species and the mixture extracted.

Antigens were detected by means of the method of precipitation in a salt-agar gel developed by Ouchterlony (22-26). This method not only makes it possible to separate the individual antigen-antibody reactions, but allows a direct comparison of antigens from different sources. This method has been used successfully with sea urchin material by Perlmann (27). Serological absorption was carried out in the manner described by Björklund (3, 4).

RESULTS

When the antisera to the parent species (48 hour embryos) were tested with the 2 M NaCl extracts of the parent species (48 hour embryos), no common antigens among the species could be found. Thus, a 2 M NaCl extract of embryos of *Paracentrotus* × *Paracentrotus* gave two strong precipitation lines and one weak line with antiserum to *Paracentrotus* × *Paracentrotus*. A 2 M extract of *Psammechinus* × *Psammechinus* gave no precipitation line with antiserum to *Paracentrotus* × *Paracentrotus* (Fig. 1). Likewise, a 2 M extract of *Psammechinus* × *Psammechinus* gave a strong precipitation line with antiserum to *Psammechinus* × *Psammechinus*, whereas a 2 M extract of *Paracentrotus* × *Paracentrotus* did not (Fig. 2). A 2 M extract of *Arbacia* × *Arbacia* gave 4 precipitation lines with antiserum to *Arbacia* × *Arbacia*, whereas 2 M extracts of *Paracentrotus* × *Paracentrotus* did not (Fig. 3). Thus, no antigen which is *common* to the species used could be detected in this fraction.

Results of testing 2 M NaCl extracts of the hybrid embryos (48 hours) were as follows (see also Table II, upper pert):

Paracentrotus ♀ × *Psammechinus* ♂: Extracts of *Paracentrotus* ♀ × *Psammechinus* ♂ gave a strong precipitation line with antiserum to *Psammechinus* × *Psammechinus*, although *Paracentrotus* × *Paracentrotus* extracts did not (Fig. 2). This indicates that as early as 48 hours (early plutei) an antigen peculiar to the paternal species has appeared in the hybrid. As was to be expected, *Paracentrotus* ♀ × *Psammechinus* ♂ extracts also gave a strong precipitation line with antiserum to the maternal species (*Paracentrotus* × *Paracentrotus*) (Fig. 1).

Psammechinus ♀ × *Paracentrotus* ♂: Extracts of the reciprocal hybrid, *Psammechinus* ♀ × *Paracentrotus* ♂, did not give such strong results, but a weak reaction could be detected with antiserum to the paternal species

(*Paracentrotus* × *Paracentrotus*) (Fig. 1). As was expected, a strong reaction with the antiserum to the maternal species (*Psammechinus* × *Psammechinus*) could be seen (Fig. 2).

Paracentrotus ♀ × *Arbacia* ♂: With extracts from the *Paracentrotus* ♀ ×

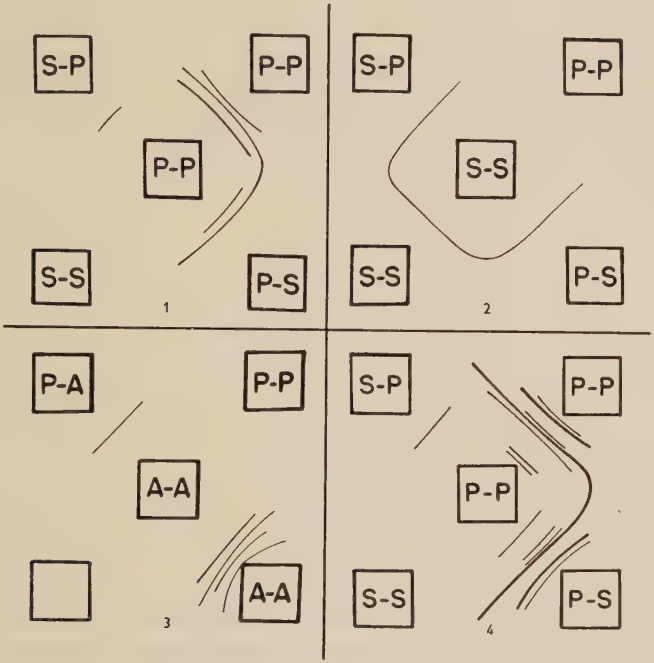

Fig. 1. Antigen-antibody reactions obtained with antiserum to embryos of *Paracentrotus* × *Paracentrotus* (48 hours) in the center well, and 2 M NaCl extracts of embryos (48 hours) in the 4 peripheral wells. P-P = *Paracentrotus* × *Paracentrotus;* S-S = *Psammechinus* × *Psammechinus;* P-S = *Paracentrotus* ♀ × *Psammechinus* ♂; S-P = *Psammechinus* ♀ × *Paracentrotus* ♂.

Fig. 2. Antigen-antibody reactions obtained with antiserum to embryos of *Psammechinus* × *Psammechinus* (48 hours) in the center well, and 2 M NaCl extracts of embryos (48 hours) in the 4 peripheral wells. Abbreviations as in Fig. 1.

Fig. 3. Antigen-antibody reactions obtained with antiserum to embryos of *Arbacia* × *Arbacia* (48 hours) in the center well, and 2 M NaCl extracts of embryos in the 3 peripheral wells. P-P = *Paracentrotus* × *Paracentrotus;* A-A = *Arbacia* × *Arbacia;* P-A = *Paracentrotus* ♀ × *Arbacia* ♂.

Fig. 4. Antigen-antibody reactions obtained with antiserum to embryos of *Paracentrotus* × *Paracentrotus* (48 hours) absorbed with *Psammechinus* × *Psammechinus* (48 hours) in the center well, and 0.14 M NaCl extracts of embryos in the 4 peripheral wells. Abbreviations as in Fig. 1.

Arbacia ♂ hybrid a weak reaction to the paternal (*Arbacia* × *Arbacia*) antiserum was detected (Fig. 3).

Using the 0.14 M NaCl extracts the results are more complicated. Extracts of *Paracentrotus* × *Paracentrotus* gave some precipitation lines in common

TABLE II

Summary of the reactions obtained. + indicates strong positive reaction, — indicates negative reaction, ± indicates weak reaction. P = *Paracentrotus*, S = *Psammechinus*, A = *Arbacia*. In all cases the egg species is mentioned first (e. g., P × S = *Paracentrotus* ♀ × *Psammechinus* ♂).

Antigen solutions	Antisera					
	P × P	P × P absorbed with S × S	S × S	S × S absorbed with P × P	A × A	A × A absorbed with P × P
2 M NaCl extracts						
P × P	+		−		−	
S × S	−		+			
A × A					+	
P × S	+		+			
S × P	±		+			
P × A	+				±	
0.14 M NaCl extracts						
P × P	+	+	+	−	+	−
S × S	+	−	+	+		
A × A	±				+	+
P × S	+	+	+	±		
S × P	+	±	+	+		
P × A	+				+	±

with extracts of *Psammechinus* × *Psammechinus*, as did the unfertilized egg extracts of the two species. *Arbacia* × *Arbacia* and *Paracentrotus* × *Paracentrotus* extracts when tested with *Arbacia* × *Arbacia* antiserum both showed precipitation lines, although the heterologous reactions were presumably due to cross reactions of non-identical components, since the lines did not fuse at the corners (see Ouchterlony, 22) and developed much slower than in the homologous reactions. The same was the case when extracts of *Arbacia* eggs and *Paracentrotus* eggs were tested against antiserum to *Arbacia* eggs. Similar results were obtained with extracts of *Paracentrotus* × *Paracentrotus* and *Arbacia* × *Arbacia*, tested with antiserum to *Paracentrotus* × *Paracentrotus*.

Because of the occurrence of common or similar antigens between the parental species in the 0.14 M NaCl extracts, little could be determined about antigen development in the hybrids from these extracts without first absorbing the antisera with the antigens of one or the other parental species. Thus antiserum to *Psammechinus* × *Psammechinus* was absorbed with *Paracentrotus* × *Paracentrotus*. After this it gave no reaction with 0.14 M NaCl

TABLE III

Control experiment. Reactions obtained when antigen consists of extract of lyophilized eggs experimentally contaminated with lyophilized sperm of another species (0.1 g lyophilized sperm / 1 g lyophilized eggs). P = *Paracentrotus*, S = *Psammechinus*, A = *Arbacia*.

Antigen solutions	Antisera						
	P × P	P × P absorbed with S × S	S × S	S × S absorbed with P × P	A × A	A × A absorbed with P × P	P × P absorbed with A × A
2 M NaCl extracts							
P eggs, S sperm	+		−				
S eggs, P sperm	−		+				
P eggs, A sperm	+				−		
0.14 M NaCl extracts							
P eggs, S sperm		+		−			
S eggs, P sperm		−		+			
P eggs, A sperm						−	+

extracts of *Paracentrotus* × *Paracentrotus*, but still reacted strongly with extracts of *Psammechinus* ♀ × *Paracentrotus* ♂ and *Psammechinus* × *Psammechinus*, and weakly with *Paracentrotus* ♀ × *Psammechinus* ♂ extracts. Another example of the results obtained with 0.14 M NaCl extracts and absorbed serum is shown in Fig. 4. Table II presents a summary of the results.

The results with extracts of *Paracentrotus* ♀ × *Psammechinus* ♂ (24 hour embryos) were identical with the results obtained with the 48 hour *Paracentrotus* ♀ × *Psammechinus* ♂ material. Likewise with *Psammechinus* ♀ × *Paracentrotus* ♂ material, 30 hour, 48 hour, and 93 hour embryos all behaved serologically alike. *Paracentrotus* ♀ × *Arbacia* ♂ (35 hour embryos) gave the same results as did 48 hour embryos of the same cross.

In the extracts of the material lyophilized one hour after sperm had been added, no paternal antigens could be detected, which indicates that the results obtained should not be explained on the basis of contamination by sperm of a foreign species. In addition we were unable to extract any antigens from the sperm of *Arbacia* and *Paracentrotus* using 2 M NaCl, although antigens could be extracted with 2 M NaCl both from the eggs and the normal embryos of these species and from the hybrids. Furthermore, in the experiments in which lyophilized egg material was mixed with lyophilized sperm from a different species in a ratio of 10 : 1, no antigens peculiar to the sperm species could be detected (Table III). As the amount of sperm substance added in this experiment far exceeds the amount of sperm added in a culture,

there seems to be little doubt that the paternal antigens detected in hybrid cultures are not due to sperm contamination but rather have developed under the influence of the hybrid nucleus some time before 24 hours, or the beginning of gastrulation.

It was possible to obtain some antiserum to one of the hybrids (*Psammechinus* ♀ × *Paracentrotus* ♂) (48 hours). By absorption experiments it was again possible to show that antigens other than maternal ones occur in the 48 hour hybrid. At present it is not possible to state whether any antigens do, or do not, occur, which are peculiar to the hybrid (hybrid substance, see for example Irwin (15)). The failure to obtain any reaction when the hybrid serum was absorbed with material from both parental species may possibly have been due to the fact that the serum was very weak.

DISCUSSION

It has long been known that interspecific hybrids often are morphologically intermediate between the two parent species. The majority of the literature is concerned with adult hybrids, or at least hybrids, which are relatively far advanced in their development. Among the studies of echinoderm embryonic hybrids are those of Hörstadius (12), E. B. Harvey (11), and A. R. Moore (20). Hörstadius, working with hybrids of *Psammechinus microtuberculatus* and *Paracentrotus lividus*, found the pluteus of 8–9 days to be intermediate in form between the two parent species. That is, he found characteristics of both the maternal and paternal species in the hybrid pluteus. The hybrid plutei obtained by Harvey (working with several crosses, among them *Strongylocentrotus purpuratus* × *Strongylocentrotus franciscanus*) were purely maternal in appearance. A. R. Moore, however, found the 4 day old pluteus of the cross *Strongylocentrotus purpuratus* ♀ × *S. franciscanus* ♂ to be intermediate in appearance.

On the other hand, from older observations of hybrid development, it is known that the early cleavage tempo is characteristic of the maternal species. For a review of this subject see A. R. Moore (19). Presumably, however, the paternal chromosomes exert some influence on development prior to the appearance of definite morphological differences.

Perlmann and Gustafson (28) have determined the presence of antigenic material in sea urchin embryos, and have found that new antigens appear after gastrulation. This has been confirmed by Perlmann (27) using the method of Ouchterlony (22–26). By extending these methods to early hybrid embryos, and the embryos of the parental species, it has been possible to

demonstrate the relatively early appearance of paternal traits, i.e. antigenic substances characteristic of the paternal species, before morphological traits can be easily detected. In the hybrid larvae of *Paracentrotus* and *Psammechinus* antigens of the paternal species were detected as early as 24 hours (late blastulae after the formation of the primary mesenchyme, see Hörstadius, 13, p. 134), and 48 hours (early pluteus), indicating that the nucleus has begun to take part in embryonic development at least by that time if not earlier.

In the case of the crosses of *Psammechinus* and *Paracentrotus* the hybrid is not lethal. This is not true, however, of the cross *Paracentrotus* ♀ × *Arbacia* ♂. This is a lethal hybrid, the embryo rarely developing beyond the gastrula stage. In 1910 Baltzer (1) described the cytology of hybrids of this cross. In the few he was able to obtain he found evidence of chromosome elimination. Recently it has been found possible to obtain over 90 per cent cross-fertilization of *Paracentrotus* ♀ × *Arbacia* ♂ (10), and also of *Psammechinus* ♀ × *Arbacia* ♂. Therefore, the problem was taken up again. Careful cytological investigation has revealed that in the majority of cases there were no nuclear abnormalities detectable until the early gastrula stage (2). At about 31 hours, the development stops in the majority of cases, and nuclear degeneration begins to appear. The embryos continue to live for some time though very few continue to develop much further. In such a lethal hybrid it is of interest to know whether the paternal chromosomes have any influence, other than a purely physical one, on the development of the hybrid. It is interesting to note in this connection that even in the lethal hybrid (*Paracentrotus* ♀ × *Arbacia* ♂) antigenic material of the paternal species can be detected.

It seems clear, therefore, that there is a specific chemical difference between the hybrid embryo and the embryo of the maternal species at a stage where there is as yet no readily observed morphological difference. Since the sperm contributes essentially only nuclear material, and in view of the specificity of the serological reactions used, these differences might be expected to arise either directly or indirectly from the nucleus.

SUMMARY

Antigens were determined in embryos of *Paracentrotus lividus, Psammechinus microtuberculatus, Arbacia lixula,* and in some embryonic hybrids of these species (*Paracentrotus* ♀ × *Psammechinus* ♂, *Psammechinus* ♀ × *Paracentrotus* ♂, and *Paracentrotus* ♀ × *Arbacia* ♂) at several stages of development. The method of precipitation in a salt-agar gel as described by Ouchterlony (22–26) was used. Antigens of the paternal species were

detected in hybrids by 24 hours of development (late blastula) as well as at 30, 35, 48 and 93 hours. Control experiments indicated that the presence of paternal antigens was not due to contamination with sperm. The possible significance of these findings is discussed in relation to the role of the cell nucleus during early embryonic development.

We wish to express our sincere appreciation to Professor John Runnström for all his help and guidance. Two of us (C. V. H. and D. H.) are especially grateful to Professor and Mrs. Runnström, and other members of the Wenner-Gren Institute, for their very generous hospitality during our two years' stay there. We should also like to thank Professor R. Dohrn, Dr. P. Dohrn, and the staff of the Zoological Station, Naples, for making our visit there a pleasant one.

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Experimental Cell Research, **6**, *211–220 (1954)*

STUDIES ON THE EFFECT OF X-RAYS ON THE BIOCHEMISTRY AND CELLULAR COMPOSITION OF ASCITES TUMORS

I. EFFECT ON GROWTH RATE, CELL VOLUME, NUCLEIC ACID AND NITROGEN CONTENT IN THE EHRLICH ASCITES TUMOR

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Received October 12, 1953

The main advantage of ascites over solid tumors lies in the fact that they represent cell populations, the composition of which can be quantitatively determined with regard to the presence of cells other than neoplastic and viable. It was decided to carry out an investigation on the effect of X-rays on various biochemical characteristics of ascites tumors and to determine simultaneously the changes induced in the cell population with respect to percentage cellular composition, cell viability, mitotic index and cell volume, and to correlate biochemical and morphological changes as far as possible. In this first study the Ehrlich ascites tumor was irradiated with an X-ray dose that inhibited mitosis completely for a certain period of time but did not kill the cells. It could be shown that the cellular composition of the tumor remains constant during this period. The synthesis of desoxypentosenucleic acid (DNA), pentosenucleic acid (PNA), and nitrogen was followed as a function of time after irradiation and correlated to changes in average cell volume.

MATERIALS AND METHODS

Mice. Hybrid albino mice, received from a commercial farm, were used. All experiments were carried out with 3–4 months old males with a body weight of 20–25 g. The animals were kept on a standard compressed diet which, as well as drinking water, was available *ad libitum* at all times.

Tumor. The Ehrlich ascites tumor was employed for all experiments. After the intraperitoneal injection of appropriate numbers of tumor cells, progressive growth in the free-cell form is obtained in 100 per cent of the mice, regardless of sex or strain. The growth characteristics of this neoplasm have been studied previously (7).

Inoculations have been carried out in the following way: Ascites was collected from animals inoculated 6–14 days earlier. The fluid was stored in the refrigerator

for 2–4 hours and in the meantime the concentration of cells has been counted in a Bürker hemocytometer while the proportion of tumor cells was established by means of differential countings on stained smears (6). Dilutions of the fluid were then made with physiological saline and 17–20 $\times 10^6$ tumor cells were injected intraperitoneally to groups of 30–80 mice in volumes of 0.2–0.5 ml.

Irradiation. 7 days after inoculation the mice had 7.6 ml ascites on an average, containing a total mean number of 995 $\times 10^6$ free tumor cells (7). They were irradiated with 1250 r (165 kV, filter 1 Al, intensity 80 r/m) over the abdomen in groups of 8, and killed at various intervals after treatment up to 48 hours. The fluid was collected from all 8 animals of each group, mixed and samples were taken for the determination of cell concentration, percentage of tumor cells, percentage of viable cells, average cell volume, total nitrogen, pentose-nucleic acid (PNA), and desoxypentosenucleic acid (DNA) by the methods described in the following.

Cellular composition of the ascites tumor. Differential counts were performed on smears that were prepared immediately after collection of the fluids, and fixed and stained according to the Papanicolaou method (13). 1000 cells were counted on each slide. The cells were classified as tumor-cells in interphase (the cytological criteria are described in ref. 6), tumor-cells in mitosis, and non-tumorous cells. Mitotic figures of all stages were counted. The mitotic index was calculated as the number of tumor cells in mitosis per 100 tumor cells. The errors of the differential countings have been studied and described elsewhere (7). The viability of the cells was judged by the method of unstained-cell counts as developed by Schrek (11).

Average cell volume. The average volume of the ascites cells was determined at various intervals after irradiation by the haematocrit method. Samples of whole ascites were centrifuged in a haematocrit tube until the volume of the sediment remained constant (about 30 min. at 3,000 $\times$ g). The ratio of sediment-supernatant volumes was thus determined. In the same fluids, the total number of cells was counted in the hemocytometer and the relative percentage of tumor cells differentially counted on stained smears. Average cell volume was calculated by dividing the volume of the sediment obtained after centrifuging 1 ml ascites by the number of cells per ml. As the non-tumorous exudate cells are generally smaller than the Ehrlich ascites tumor cells (6), an increasing admixture of such cells would tend to lower the average volume. The tumor cell percentage in the material used was constantly about 85 per cent, showing but slight random variations. A 15 per cent admixture of non-tumorous exudate cells lowers somewhat the average cell volume, and the volume of tumor cells alone would be somewhat higher than the values actually obtained. This systematic error can be assumed to be similar in all samples, however, and is to a certain degree compensated by the serum trapped in the sediment and by the admixture of a small number of erythrocytes. Grossly hemorrhagic samples were discarded, as larger numbers of red cells would vitiate the data.

Nucleic acid and nitrogen content. The average amount of pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) per cell was determined by the method of Schmidt and Thannhauser (9) and that of Schneider (10) on samples of ascites containing known numbers of cells. Reasonable agreement was obtained between the two methods. The mean value was divided by the number of cells in the sample, and is interpreted to represent the nucleic acid content of the average cell. It has been shown previously (5) that the cell-free ascitic fluid does not contain nucleic

acids. The case was different for nitrogen, which was determined by a micro-Kjeldahl procedure, as the ascitic supernatant is rich in proteins and other N-containing compounds. Here the procedure was the following: One aliquot of the whole ascites fluid was used for the determination of total N per ml, a second aliquot was taken for cell counting, and the volume ratio of sediment to supernatant was established on a third sample in the haematocrit tube. The remaining part was centrifuged and the amount of N was determined in the cell-free supernatant. Knowing the proportion of supernatant for 1 ml whole ascites and its N-content, its contribution to the N-content of the whole fluid could be calculated and subtracted. The remaining value represents the N-content of the ascites cells. Average amounts per cell were again calculated. As any major admixture of erythrocytes would have vitiated the results, grossly hemorrhagic fluids were excluded from these determinations.

RESULTS

a) *Cell viability and cellular composition of the peritoneal fluid*

The percentage of eosin-stained cells is low during the entire course of growth of the Ehrlich ascites tumor (7). After irradiation with 1250 r, the number of stained cells did not increase to any perceptible degree during the following observation period of 48 hours. Neither was there any change in any of the following measures: number of cells per ml, percentage of tumor cells, total number of tumor cells in the entire peritoneal fluid. (For method of determination of the latter see ref. 7.)

b) *Mitotic index*

The mitotic index began to fall immediately after irradiation and showed an approximately exponential decrease (Fig. 1). Within 2 hours mitotic figures disappeared completely or almost completely (Fig. 2) and did not reappear until 20–30 hours later. Mitotic indices within the range of the control values were found 48 hours after irradiation. There were many abnormal mitoses present, however, and in cases where the mitotic index was followed for longer periods, a subsequent secondary decrease of the index could be observed.

When the total number of tumor cells was determined during the 48-hour period after irradiation in the entire peritoneal fluid by methods described elsewhere (7), it was found that this number remained unchanged during this interval, while considerable increase occurred in unirradiated controls. This is in agreement with the determinations of mitotic index and of cell viability and indicates that the cell population remains essentially the same during this time, and no detectable numbers of cells are disappearing or being newly formed.

c) *Average cell volume*

Microscopical examination of the smear preparations suggested that an overall increase of cell volume occurs during the period following irradiation, concomitantly with the absence of mitoses. This impression was fully

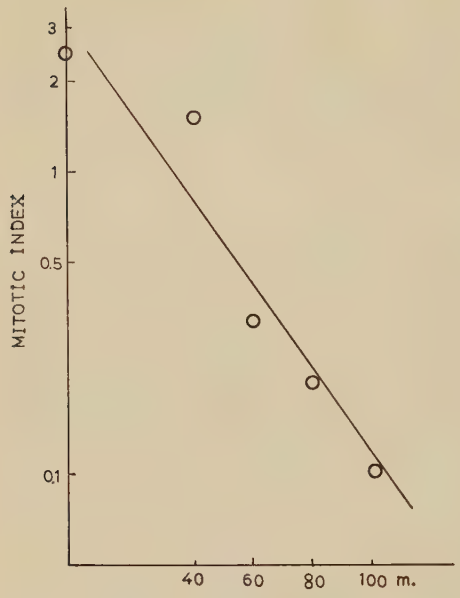


Fig. 1. Changes of the mitotic index during the first 100 minutes after irradiation with 1250 r.

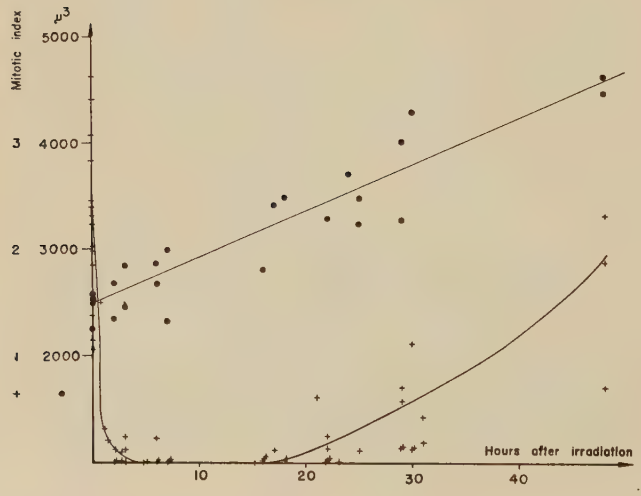
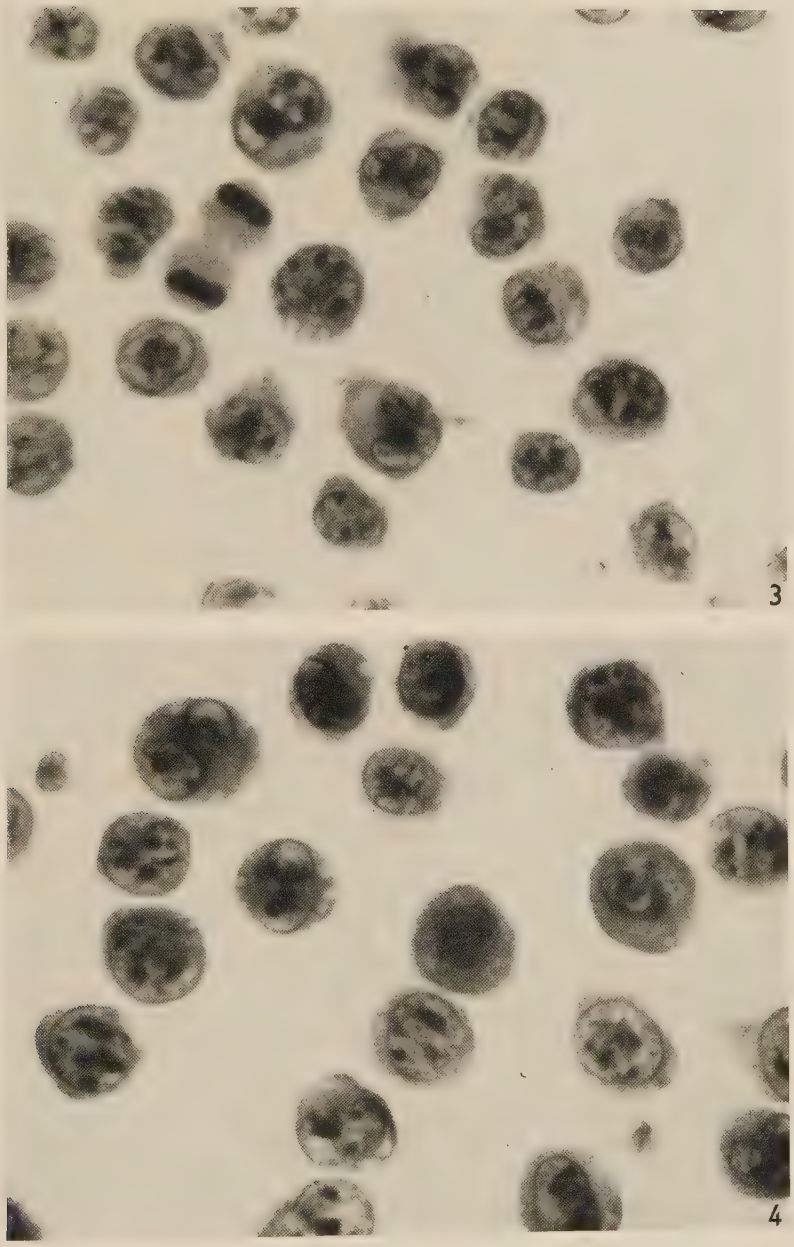


Fig. 2. Changes of the mitotic index (+) and of average cell volume (●) during the 48-hour observation period after irradiation with 1250 r.



Figs. 3 and 4. Smears of ascitic fluid, fixed and stained according to the method of Papanicolaou. Magnification × 1,000. Fig. 3: before irradiation. Fig. 4: 48 hours after irradiation with 1250 r. Note increase in cell size.

verified after having determined the average cell volume by the haematocrit method. As seen from Fig. 2, a progressive increase of volume starts immediately after irradiation and continues as an approximately linear function of time. A straight line was fitted to the experimental points by the method of least squares. A high correlation was obtained ($r = 0.9279$) and the regression coefficient indicated that the volume of the average cell increases by 43.6 ± 3.6 μ^3 per hour, i.e. about 1.75 per cent of the original value (Table I).[1]

TABLE I

Relation between time after irradiation (in hours) and average cell volume, nucleic acid and nitrogen content of the Ehrlich ascites tumor

Relation	Number of detns.	Control value	Regression coefficient $b \pm \sigma_b$	Correlation coefficient r	Significance (P)	Relative increase per hour, per cent of control value
1	2	3	4	5	6	7
Average cell volume/time	25	2 485 (μ^3)	43.6 ± 3.6	0.928	<0.001	1.75
Total nucleic acid per cell/time	13	51.2 ($\mu\mu$g)	0.621 ± 0.11	0.855	<0.001	1.20
PNA per cell/time	10	36.4 ($\mu\mu$g)	0.52 ± 0.09	0.911	<0.001	1.42
DNA per cell/time	10	16.4 ($\mu\mu$g)	0.077 ± 0.036	0.600	$0.1 > P > 0.05$	0.47
Total nitrogen per cell/time	9	47.7 ($\mu\mu$g)	0.65 ± 0.21	0.832	$0.01 > P > 0.001$	1.35
Percentage growth rate per hour $\left(\frac{dN}{dt} \cdot \frac{100}{N}\right)$ of the tumor-cell population immediately before irradiation						1.44

The microscopical examination indicated (Figs. 3 and 4) that both nucleus and cytoplasm contributed to the enlargement of the cells. There was an apparent increase in the number and volume of nucleoli.

[1] The values obtained 48 hours after irradiation may be slightly lowered due to the fact that mitoses have reappeared by this time. The decrease produced by this factor can be calculated to be 14 per cent at its maximum, and could therefore not have influenced the slope of the present regression line to any significant degree.

d) *Nucleic acid and nitrogen content*

The results are shown in Fig. 5, and the pertaining regression and correlation data in Table I. A progressive and approximately linear increase occurred in pentose nucleic acid as well as in nitrogen content during the 48-hour observation period after irradiation with 1250 r. Desoxypentosenucleic acid showed only a very slight increase. As shown in col. 6, Table I, the increase is statistically significant for total NA, PNA and N, and statistically probable for DNA.

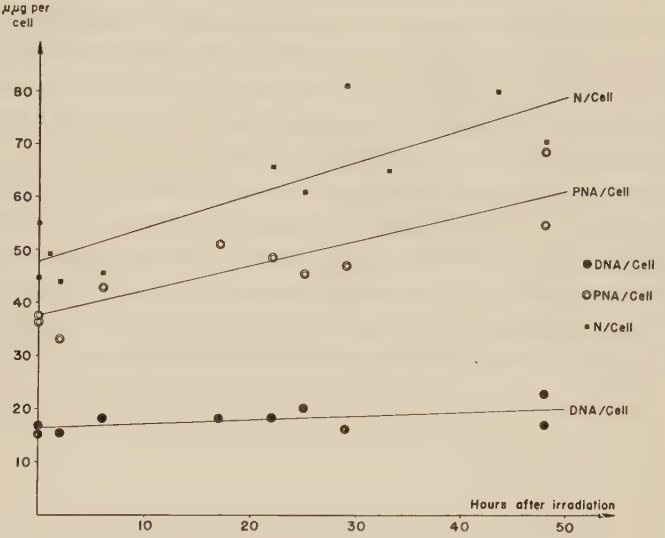


Fig. 5. Increase of the average amount of PNA, DNA, and total nitrogen per cell after irradiation with 1250 r. The straight lines are fitted by the method of least squares.

Calculating the regression coefficient as per cent of the control value obtained from unirradiated cells, a measure is obtained indicating the relative increase per hour and per cell of the chemical constituent studied or of cell volume (col. 7). It appears that average cell volume increased by 1.75 per cent of the control value per hour in the irradiated samples. This was nearly paralleled by the increase of pentose nucleic acid and nitrogen, where the increase was 1.42 and 1.35 per cent, respectively. The difference between the three regression coefficients is not significant (when comparing cell volume and PNA, $t = 1.28$, $P \sim 0.2$). Thus the amount of PNA and N per unit cell volume remained nearly constant during the period of progressive cellular enlargement that followed irradiation. Desoxypentosenucleic acid showed an entirely different behavior. It increased by only 0.47 per cent per hour and did thus not follow the other cellular constituents studied. Its concen-

tration per unit volume decreased in the enlarged irradiated cell. The difference between the percentage increase of PNA and DNA per cell is significant at the 1 per cent level ($t = 2.8$, $P \sim 0.01$).

It was of interest to compare the rate of postirradiative cellular enlargement and synthesis of PNA and N with the rate of cell multiplication in the untreated Ehrlich ascites tumor. The growth characteristics of this neoplasm have been studied previously in detail and an expression has been developed to describe the relative growth rate (equation 11, ref. 7). Inoculum dose was generally about 17–20 million tumor cells in the present experiments and the developing ascites tumor was irradiated 7 days after inoculation. Using these data and the previously developed equation, it could be calculated that immediately preceding irradiation the tumor cells were growing at a relative rate of about 1.44 per cent, i.e. an average of 1.44 new cells was formed per hour for every 100 tumor cells. This figure is surprisingly close to the postirradiative increase rates obtained for cell volume, pentose-nucleic acid and total nitrogen, indicating that the synthesis of new cell substance continues with undiminished intensity after irradiation, and, being unable to divide, the cells have to increase their volume. This holds true as far as PNA and N are concerned, while the synthesis of new DNA seems to have been inhibited by irradiation.

DISCUSSION

Irradiation with 1250 r X-rays inhibited mitosis completely for a considerable period of time in the Ehrlich ascites tumor, but did not result in any detectable increase of the percentage of dead cells during the 48-hour observation period, as judged by the method of supravital staining of Schrek (11). This is in marked contrast to findings on some other ascites tumors. In the experiments of Tarleton *et al.* (12) on the ascites form of Sarcoma 37, those of Freymann (1) on the Yoshida ascites sarcoma, and our own unpublished work on the ascites form of DBA lymphoma, irradiation with 800, 400, and 1250 r, respectively, produced a pronounced reduction of the concentration and percentage of free tumor cells and their viability. This indicates that the cells of the Ehrlich ascites tumor are comparatively radio-resistant. Also, it would appear that irradiation of ascites tumors leads to a decrease of tumor-cell percentage only in connection with cell death. A relative increase in the number of non-tumorous exudate cells occurs under these conditions, which is either due to inflammatory processes provoked by the disintegrat-

ing tumor cells, or/and to a decrease in tumor-cell concentration. Irradiation with 1250 r does not induce an increase in the number of non-tumorous exudate cells in the absence of tumor-cell death, however. This circumstance was very fortunate for the present investigation, because it enabled the biochemical study of a *tumor-cell population of constant and known composition* at various intervals after irradiation. This constancy of the cell population, suggested by the simultaneous absence of mitosis, detectable cell death and inflammatory response of the host, was directly confirmed by determinations of the total tumor-cell number in the entire peritoneal fluid which was shown to remain unchanged during the observation period.

The main result that has emerged from this first study shows that the irradiated tumor cells continue to synthesize new cell substance at about the same rate as before irradiation. They are unable to divide, however, and are increasing in size accordingly. The same phenomenon has been reported by various authors on different other biological materials (8). Nitrogen and PNA, when expressed as average amounts per cell, show about the same rate of increase as cell size, indicating that real synthesis of new cell substance occurs and not merely a swelling due to water-uptake. A different behavior is exhibited by DNA which increases to a very slight degree only, and its cellular concentration thus decreases considerably. This suggests a selective action of radiation on the synthesis of DNA which is in agreement with the findings of Hevesy, Holmes, Kelly, and others (2, 3, 4).

SUMMARY

The Ehrlich mouse ascites tumor was irradiated *in vivo* with 1250 r X-rays. This dose inhibited mitosis completely for about 15 hours but did not increase the death rate, as judged by supravital staining. A linear increase of average cell volume against time followed the treatment and resulted in a doubling of size in about 48 hours. Both nucleus and cytoplasm contributed to this increase. The rate of cellular enlargement was approximately equal to the growth rate of the cell population before irradiation, the difference being that growth after irradiation occurred mainly as increase in size of the individual cells, while in the untreated population it is solely due to cell multiplication.

After irradiation, the average amount of PNA and total nitrogen per cell increased at nearly the same rate as cell volume, i.e. their cellular concentration remained about the same. In contrast, the amount of DNA showed

only a very slight increase, resulting in a decrease of cellular concentration of this compound. This indicates a specific action of irradiation on the synthesis of DNA.

This work was made possible by a grant from the Swedish Anti-Cancer Society.

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Experimental Cell Research, **6**, 221–227 (1954)

INDUCTION OF SYNCHRONOUS CELL DIVISION IN MASS CULTURES OF *TETRAHYMENA PIRIFORMIS*

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Received October 22, 1953

With the ultimate scope of making the mitotic cycle accessible to the study with standard chemical and physiological techniques, we have during the past year endeavored to produce synchronism of cell division in mass cultures of *Tetrahymena piriformis*, Lwoff's strain.

Tetrahymena was selected because it can be grown in pure culture in proteose-peptone media and—sole among animal cells—also in fully defined media as reported by Kidder and Dewey (4). One possible way of synchronizing the growth activities of this organism would be to place a barrier at a certain point in the cell cycle, say by using inhibitors known or suspected to inhibit specific phases. Colchicin and Aminopterin were tried, with meso-inositol (5) and folic acid, or folic acid derivatives, as suggested releasing agents. We failed because the inhibitors proved non-toxic, as earlier reported (3, 4).

The effect of temperature has been tried, however, starting out from a different theory. Ephrussi (2) many years ago reported that the Q_{10} is different for the different phases of cell division in the sea urchin egg. It was, therefore, considered that exposure to lowered temperature, for a time shorter than the mitotic cycle at the low temperature, with subsequent return to optimum temperature, might tend to bring closer separate stages having different Q_{10}'s. After the lapse of another cycle at optimum temperature repetition of the treatment should tend to make the group larger, etc., till synchronism in the whole culture had been produced. Indeed, shifts of temperature $28° \rightleftharpoons 7°$C had some effect, but disturbing factors seemed to interfere. These experiments, however, led the way to successful experiments in which the temperature was periodically raised to sublethal levels. This paper reports such experiments. We are not yet going to offer full interpretation of our findings; we think, however, that the results are best understood, not on the basis of different Q_{10}'s as discussed above, but on

[1] Supported by a grant of "Statens Almindelige Videnskabsfond".

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the basis of the assumption that sublethal temperatures act by blocking —with a considerable recovery time at optimum temperature—a specific step in the cell cycle.

EXPERIMENTS

The cells were grown as pure cultures in 2 per cent proteose peptone (Difco) + 1 per mille liver fraction L (Wilson Laboratories); salts were added according to Kidder and Dewey (4). Under conditions of ample supply of oxygen, in single cell cultures grown in capillaries on 0.5–1.5 μl, medium population densities may reach 1.2 million cells/ml on this medium (personal communication from Mr. H. Thormar). However, there is evidence that true exponential multiplication does not continue beyond population densities of about 100,000 cells/ml or perhaps less. In the experiments to be reported we have therefore worked with very dilute cultures (5,000–50,000 cells/ml), offhand assumed to be in the log-phase of growth. We used two types of culture flasks: either salt cellars (1 ml culture medium, covering 4.5 cm^2, average depth 2.2 mm, bottom slightly hollow) or $^1/_2$ l, flat-bottomed flasks (10 ml culture medium covering 57 cm^2, depth 1.75 mm). In the salt cellars the glass walls are thicker (5–6 mm) than in the big flasks (about 1 mm). No shaking was used. The flasks were dipped into a water bath which was regulated to $\pm$ 0.1° C. The temperature of the bath could be shifted up and down by the use of a signal watch which at predetermined times switched the current from one thermoregulator to another. Adjustment of the temperature in the bath always occurred with some delay (8 min. for an increase from 28° to 34° C, and 12 min. for a drop from 34° to 28° C), and within the cultures themselves the delay was in addition influenced by the amount of culture medium and by the thickness of the glass. The figures corresponding to those given for the bath were 20, resp. 17 minutes for the salt cellars, and 15 resp. 14 minutes for the $^1/_2$ l flasks. Usually, cultures were inoculated from stationary phase cultures (kept at 23–24° C) and grown at 28° C; after the end of a lag the division index was followed; it represents the ratio between number of cells in division and number of cells not dividing. As division cells were counted all stages from the onset of cytoplasmic constriction to separation of the daughter cells. The division index was determined on 100 μl samples fixed in 5 per cent formaldehyde; every point represents the counting of about 300 cells. We found the division index to vary from about 0.05 to about 0.10. Single cells growing in 0.5–1.5 μl of the medium here used were observed to divide every 135 minutes at 28° C ($\pm$ 20 min.). Division times of 6.8–12.5 min. have been reported (1). The division index calculated from the two set of data is 0.05–0.095, which corresponds nicely with our observations (cf. Fig. 1).

Upon transfer from 28°–29.5° C (which shall hereafter, without special qualification, be called "optimum temperature", cf. however (6)) to a sublethal temperature (32°–34° C)) the division index tends to decrease and soon it gets very low. However, at the high temperature there is a partial or a complete block for growth (6). So we suggest that sublethal temperature

prevents growth (e.g. syntheses), it also prevents new cells from entering a division, but it does not prevent a division, once initiated, from running to completion.

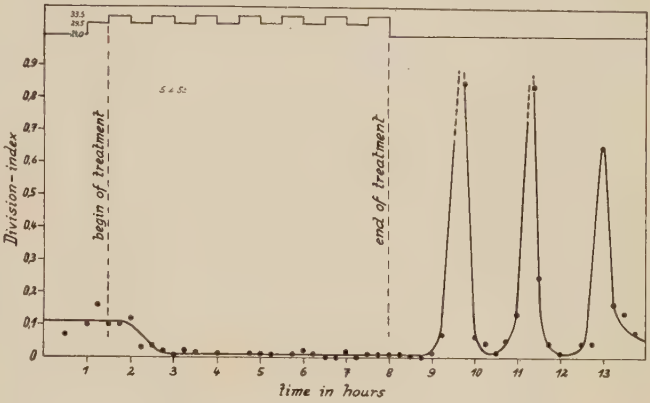

Fig. 1.
Division index in a culture of *Tetrahymena piriformis* treated for $6^1/_2$ hours with intermittent heat shocks. The uppermost curve shows the switch of the temperature as indicated by signals on the watch, respectively transfer into bath (1 h) and removal from bath (8 h). Proteose - peptone culture, 10 ml in flask. Total initial population about 400,000.

However, we expose cells to sublethal temperatures only for a short time and then, for another short period, we transfer them to the optimum temperature. The cells then resume growth but they do not, within this short time, begin to divide. Before they recover the mechanisms which in normal cells operate to switch cells from synthesis (growth in mass) to division they are exposed to another short temperature shock and thereafter again transferred to optimum temperature and so on. With this treatment of intermittent heat shocks (we might say: "intermittent fever") the cells grow bigger and bigger (cf. Fig. 2 a, b). After several hours they are returned to constant optimum, or usually just to room temperature (the dishes were placed under a binocular microscope for continuous observation). About $1^1/_2$ ($\pm$ 10 minutes) hours at this temperature a burst in division activity was observed (Fig. 2c). As many as 85 per cent of the cells may be in division simultaneously. From now on, through a few mitotic cycles, the divisions appear to be synchronized (cf. Fig. 1). We have observed three successive peaks, with respectively 85, 83 and 64 per cent of the cells dividing simultaneously. In between there are minima in which only about 2 per cent of the cells are in division.[1] The peaks appear to be closer in time than indicated by the duration of the normal mitotic cycle (at 24° C: 1.7 hours, as compared with a normal of 2.5–3 hours).

[1] Here we may add that in the experiments with lowered temperature we found a low first peak in division activity, but after this division rate returned, more or less, to the average for an untreated culture, e.g. no rhythmicity was induced.

Obviously, in the type of experiment reported there is a considerable number of variables which have not yet been examined in sufficient detail. In attempting to establish conditions of temperature treatment which will produce the highest possible degree of synchronism in the cultures we have made the following observations:

Time of treatment: 6–10 hours. Shorter treatment tends to produce lower peaks of division activity and longer treatment (16 hours) results in expressed distortion of the cells.

Temperature during treatment: so far we have obtained the best results if we let the temperature shift between 29° and 33° C (32.3°–33.7° C in the different cases) with a period of 1 hour, e.g. $^1/_2$ hour high, and $^1/_2$ hour low. Two-hour periods were rather ineffective, the division index was not considerably reduced during treatment, and not very high after. Thirty-minutes periods were more effective than 2-hours, but still inferior to 1-hour periods. We have also tried to change the ratio: time at high temperature over time at low temperature, however, with no beneficial effects. Increasing the ratio tended to make the cells more distorted, decreasing the ratio resulted in cell multiplication during treatment and in less piling up of divisions.

During the heat treatment the average cell size increases, e.g. about 3-fold in 7–8 hours. This is illustrated in the photomicrographs, Fig. 2 a, b, c. The treated cells (b) become big and, compared with the controls (a), relatively thick (pear-shaped). Also, they may become somewhat distorted. In addition to what has been said above we add that for a minimum of distortion to develop it may be essential that 1) the population density is so low (order 5,000–50,000/ml) that true exponential multiplication would have been possible had we not exposed the cells to temperature treatment, that 2) oxygen is not limiting in any part of the culture, and 3) that the temperature changes are not too abrupt. Even if there are some distorted cells by the end of heat treatment, all cells assume a perfectly normal look after one or two cycles at optimum or room temperature. It should be commented that no—or at least very few—cells are killed either during or after the heat treatment. Actually, during treatment cell numbers increase very slowly, and about as much as could be expected from the very low, however real, division index found during treatment.

The increase in cell size was followed by volume measurements of single cells in a compression chamber a.m. Scholander, Claff and Sveinsson (7). While the average cell volume increases as shown in Fig. 3, curve ×, the relative size differences, initially observed, are retained. The average protein content was studied with an unconventional (and not yet well enough

Fig. 2. Exp. 29. 4. 1953. Proteose - peptone culture, 10 ml in flask. Total initial population 115,000 cells. Individuals alive when pictures taken.
a: untreated culture (temp. 24° C) several hours after inoculation.
b: cells treated 9 hours at 28° ⇌ 34° C ($^1/_2$ hour high, $^1/_2$ hour low). Photographed $^1/_2$ hour after return to 24° C.
c: same after 100 minutes at 24° C.

checked) method in which the amount of fixative picked up by cells fixed (for 3–5 days, maximum weight after 3 days) in 3 per cent phosphotungstic acid was measured as the reduced weight of the fixed cells. A Cartesian diver balance (8) floating in the fixative was used. The growth during heat

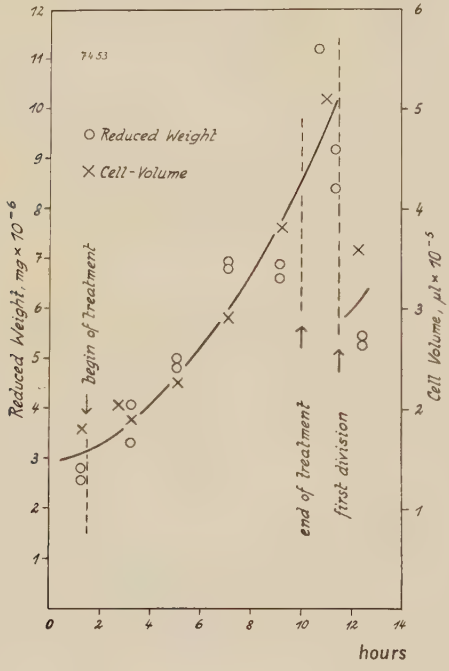

Fig. 3.
Reduced weight (RW) per cell, after 7–8 days of fixation in 3 per cent phosphotungstic acid. RW measurements carried out in the fixative (density: 1.0226, water at same temperature 1.0000). RW of unfixed cells not measured; however, cells fixed for 4 hours weigh only 40 per cent of same cells fixed for three days or more. × cell volumes measured as area × height in a compression chamber, height 10 µ.

treatment is not only cytoplasmic; also the macronuclear volume increases very considerably. After recovery from heat treatment, e.g. at optimum or room temperature, the cells tend to become smaller with each new maximum of division. That is, now the situation is reversed relative to the one during heat treatment: cell divisions are outbalancing the growth activities. For *Tetrahymena* it has previously been reported (9) that big cells can be harvested from stationary phase cultures. Upon transfer to fresh medium these cells resume growth, but they divide faster than they double in mass, with the result that in the early generations cells get smaller and smaller. Carefully traced growth curves for single cell cultures (9) were interpreted to indicate that the growth in mass (mass measured by the amount of respiration!) does not keep up with the divisions because the initial big cell grows exponentially with only part of its body; the remainder does not grow at all

and becomes diluted away upon the increasing number of cells in the progeny. It is a situation like this which we believe pertains to the giant cells produced by the heat treatment.

SUMMARY

This preliminary report shows that, using intermittent heat treatment of mass cultures of *Tetrahymena*, 85 per cent of the cells can be induced to undergo division simultaneously. Three successive maxima of divisions have been observed. The cell populations used represent total cell numbers of anything between 5,000 and $^1/_2$ million corresponding to dry weights of the order 0.03 to 5 mg. The work is continued with attempts to synchronize much larger quantities of material, first on proteose-peptone media, then on synthetic media.

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FREEZE-DRYING WITH A MODIFIED GLICK-MALMSTRÖM APPARATUS

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Received October 30, 1953

The freeze-drying procedure is a valuable method to preserve biological material for histo- and cytochemical investigations. By means of initial rapid freezing of the tissue samples, followed by dehydration in the frozen state and subsequent embedding, thin sections of tissues can be prepared with a minimum of structural and chemical changes (2, 22). Various techniques regarding the different steps in the freeze-drying procedure have been reviewed by several authors (i.a. 2, 6, 7, 10, 12, 16, 20). Highly efficient dehydration has been obtained in equipment with a water condenser at the temperature of liquid nitrogen close to the specimens combined with a high vacuum (9, 14, 21). Based upon theoretical considerations (13) Glick and Malmström (8) have devised an efficient freeze-drying apparatus of this type with remarkably short dehydration time.

In the present paper the freeze-drying procedure with a modified Glick-Malmström apparatus and a new technique for the embedding *in vacuo* will be communicated.

DESCRIPTION OF APPARATUS AND TECHNIQUE

In the following the three main steps of the freeze-drying procedure, the initial freezing, the dehydration *in vacuo*, and the embedding technique, will be considered separately. The main features of importance for the choice of apparatus and technique will be briefly discussed.

Initial freezing.

In order to avoid postsampling artefacts such as ion diffusion and enzymatic decomposition (1), as far as possible, it is necessary to freeze the tissue samples instantaneously after their removal from the organism. Ice crystal formation within the specimens is the main cause of structural changes occurring during the freezing procedure. A high rate of ice crystal nucleation, and a low rate of growth of the ice crystal nuclei, give the smallest crystals possible, and thus minimal structural changes. As the rate of for-

Fig. 1.

Fig. 2.

Fig. 1. Propane container for the initial freezing of the tissue samples.
Fig. 2. Tissue sample holders.

mation of new ice crystal nuclei is increased by a factor of 10^{18} between —33° C and —43° C (4), the tissue samples should be frozen as rapidly as possible, well below this range of temperature. A freezing medium with a high conductivity and a very low temperature is therefore necessary. A medium such as liquid air (5) has a sufficiently low temperature, but a layer of vaporized air forms an envelope around the specimens, resulting in poor conductivity (17). Isopentane, cooled by liquid nitrogen, has a high conductivity (11) but solidifies at about —150° C. Pure propane has a still higher conductivity (3) and remains fluid at —185° C, and is therefore very useful for the initial freezing. In order to obtain rapid initial freezing of the inner parts of the specimens, these must be small. When too large pieces of tissue are frozen, formation of ice crystals of considerable size will occur which causes structural changes in the inner layers of the tissues (19). The transport of the frozen specimens from the cooling medium to the dehydration chamber must be quick enough to prevent an increase of the temperature to the range where the growth of the ice crystals is accelerated (at about —40° C).

Liquid propane was used for the initial freezing of the tissue samples. A metal container with an external loop for the condensation of the propane

was devised (Fig. 1). It was placed in a Dewar flask filled with liquid nitrogen. When the container had obtained adequately low temperature, propane gas was let through the loop, where it condensed and then dropped into the container. Immediately after the removal from the organism small pieces of tissue (with an extent of less than 2 mm in one dimension) were plunged in the fluid propane. The tissue pieces were allowed to sink free to the bottom of the container, thus continuously being surrounded by fresh volumes of the cooling medium. The use of forceps or of holders for the individual specimens was avoided because of the risk in delaying the freezing process. A small metal basket placed in the bottom of the container served to make it easier to collect the samples for further transport.

The tissue sample holders were made of solid iron in which grooves for the individual specimens were drilled (Fig. 2). The grooves were filled with paraffin, degassed at 10^{-2} mm Hg for 30 minutes in a separate simple vacuum unit. Subsequently the tissue holders were placed in the sample tube (see below) which was cooled by a carbon dioxide - acetone mixture in a Dewar flask. The frozen tissue pieces were transferred very rapidly from the propane container to the cooled holders in the sample tube by means of thin forceps, cooled in the liquid propane before touching the specimens.

Dehydration.

The temperature of the specimens and of the water condenser, the distance between the samples and the cold trap, and the magnitude of the vacuum (pumping speed), are the important factors to be considered in a freeze-drying equipment with a water condenser at low temperature.

Different temperatures of the samples during the dehydration procedure have been suggested (5, 12, 15, 17, 18, 21). As insufficient physical data are available, this temperature cannot be selected theoretically (2). Above a sample temperature of —40° C, morphological changes probably due to recrystallization, are demonstrable in the tissues (19). Theoretically the dehydration temperature should be kept below the eutectic point for the salt systems present in the tissues (about —55° C). Otherwise ion diffusion may occur. As low a temperature as —60° C would be safe also for highly diffusible ions (8).

If the time for dehydration at such a low temperature is to be reasonably short, a water condenser with very low temperature, placed close to the specimens, and a high vacuum in the system are required. Liquid nitrogen provides sufficiently low water vapor tension in the trap (10^{-24} mm Hg).

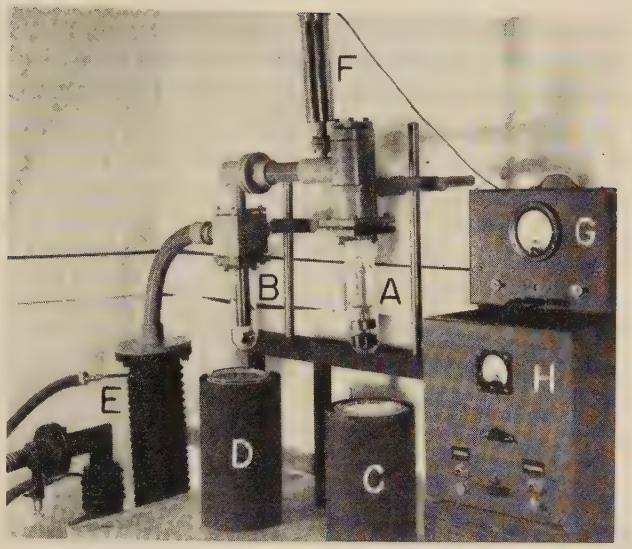


Fig. 3. View of the freeze-drying apparatus.
A. Sample tube with the close cold trap.
B. Distant cold trap.
C. Dewar flask for carbon dioxide - acetone mixture.
D. Dewar flask for liquid nitrogen.
E. Oil diffusion pump.
F. Pirani vacuum gauge.
G. Vacuumeter.
H. Oscillator.

A short distance (less than 4 cm) between the samples and the trap accelerates the removal of water from the specimens (8). Further, a high vacuum (of the magnitude of 10^{-5} mm Hg) increases the mean free path of water molecules, and therefore shortens the time for dehydration (13).

According to these principles Glick and Malmström (8) have devised an efficient freeze-drying apparatus consisting of a Pyrex glass unit with a close and a distant cold trap and a high vacuum system. However, the use of liquid nitrogen in the traps in combination with the high vacuum was considered to be hazardous because of the fragility and risk of explosion of the rather complex glass unit.

Therefore, a modified dehydration apparatus was built in metal with a minimum of glass parts (Fig. 3). The vacuum system consists of a three stage oil diffusion pump (Distillation Products Type MC 275) with a capacity of 275 l/sec. at 10^{-4} mm Hg, backed by a two stage rotary mechanical fore-pump (Pfeiffer No. 3006) with a capacity of 5 m^3/hour.

A detailed diagram of a slightly changed model is shown in Fig. 4. The apparatus is built in metal except for the cooled parts, consisting of the tubes *A*, *B*, and *G*, which are made of Pyrex glass. The metal parts are connected either by solders or by means of O-rings (G. Angus and Co. Ltd., Newcastle upon Tyne) into a vacuum-tight unit. The glass tubes *A*, *B*, and *G* and the stainless steel tube *F* are attached to the metal blocks *C* resp. *E*. These connections are made vacuum-tight by means of a system of metal discs

and O-rings, as shown in the figure. The system permits an adjustment in height of the close cold trap tube *B* as well as of the steel tube *F*. The two blocks *C* and *E* are mounted in a stable stage. The tombac tube *H* and the adjustability in height of the tube *F* make small adjustments in the distance between the two blocks possible. All vacuum leads are wide enough to permit a high pumping speed.

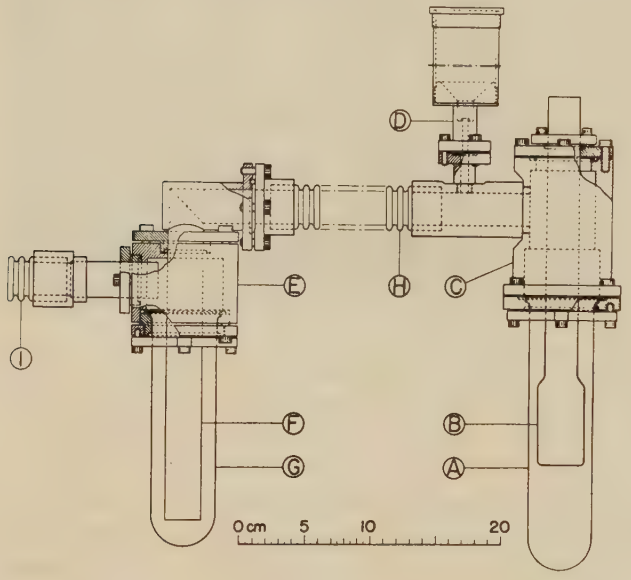


Fig. 4. Diagram of the freeze-drying unit.
A. Sample tube (Pyrex glass).
B. Close cold trap tube (Pyrex glass).
C. Metal block to connect tubes *A* and *B*.
D. Holder for the Pirani vacuum gauge.
E. Central metal block in the distant cold trap.
F. Central tube of stainless steel.
G. Distant cold trap tube (Pyrex glass).
H. Tombac tube.
I. Tombac tube connection to the oil diffusion pump.

A complete view of the freeze-drying apparatus is shown in Fig. 5. In this model the vacuum pumps consist of a two stage oil diffusion pump (Kylteknik, Stockholm, No. 2560) with a capacity of 500 l/sec. at 10^{-4} mm Hg, and a two stage rotary mechanical fore-pump (Kylteknik, Stockholm, No. 2500) with a capacity of 5 m^3/hour.

Before the dehydration was started the distant cold trap tube was surrounded by liquid nitrogen in a Dewar flask. Surrounded by the carbon dioxide - acetone mixture in a Dewar flask, the sample tube with the tissue holders carrying the specimens was attached to the apparatus. The close cold trap was filled with liquid nitrogen. Refilling was required every 4 hours. High vacuum (about 10^{-5} mm Hg) was obtained in 15 minutes with the vacuum pumps used. Initial dehydration with the sample tube cooled below —60° C was performed for 6–12 hours, depending upon the size of the tissue pieces and the nature of the material. Subsequently the Dewar flask

with the carbon dioxide - acetone mixture was removed, and the sample tube was allowed to reach room temperature. Simultaneously the liquid nitrogen in the close cold trap evaporated. In general the dehydration was now continued for 8–12 hours (over night) with the samples at room temperature and the distant cold trap surrounded by liquid nitrogen (refilling required every 12 hours). The moisture initially condensed on the walls of the close cold trap was then rapidly transferred to the distant cold trap. Subsequently, addition of liquid nitrogen to the close cold trap did not result in condensation of visible moisture if the dehydration was complete.

Various tissues required different times for the initial dehydration at a temperature below —60° C. For pieces with a maximal thickness of 2 mm the following approximate times have been found sufficient:

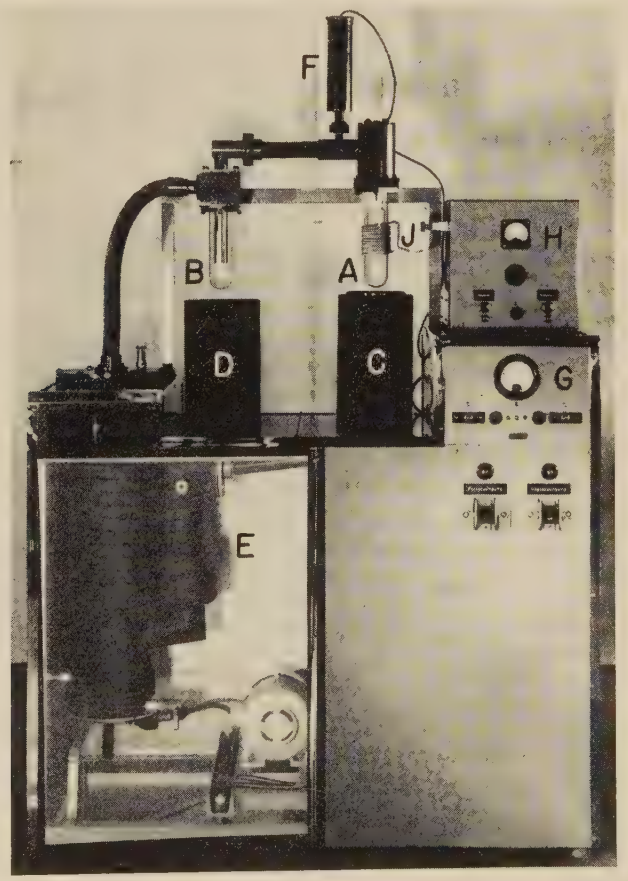


Fig. 5. View of the freeze-drying apparatus (later modification).
A–H. The same as in Fig. 3.
J. Copper wire loop (the outer inductance of the oscillator).

Tissues with a moderate water and lipid content (e.g. epithelial tissues, liver, pancreas) . . . . . 6 hours
Tissues rich in water (e.g. intestinal mucosa, testis) . . . . 8 hours
Tissues with a high lipid content (e.g. adrenals, renal parenchyma, cerebral cortex) . . . . . 12 hours

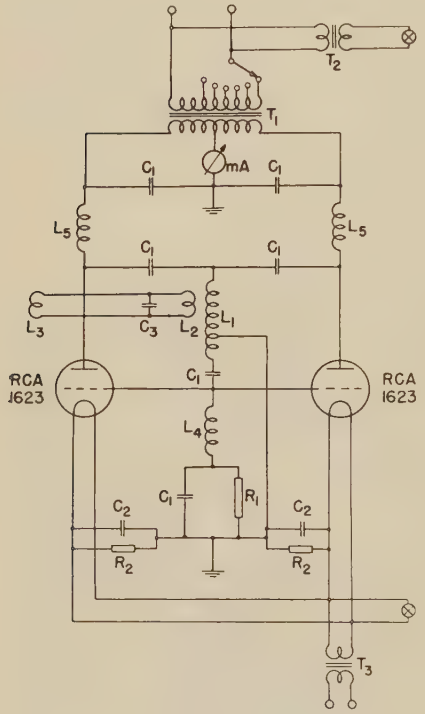


Fig. 6. Wiring diagram of the oscillator circuit.
T_1. Transformer, 220/500–1000 volts.
T_2. — 220/8 volts.
T_3. — 220/6.3 volts.
L_1. Inductance, ~100 μH (air-winded coil.)
L_2. — (Coil, diam. 90 mm. Copper wire, 5 × 3 mm, 6–8 turns.)
L_3. — (Coil, diam. 55 mm. Copper wire, 3.5 × 2 mm, 11 turns.)
L_4. — 2.5 mH (choke coil.)
L_5. — 4.3 mH (choke coil.)
C_1. Capacitance, 5000 pF.
C_2. — 10000 pF.
C_3. — 1000–1500 pF.
R_1. Resistance, 20 kΩ.
R_2. — 50 Ω.

Dehydration at temperatures close to —40° C is less time-consuming but has not given reliable results. Initial dehydration for a limited time (minimum 6 hours) with a temperature of less than —60° C followed by drying at higher temperatures (—30° C to —40° C) gave no demonstrable tissue changes. The total time for complete dehydration could thus be shortened considerably.

Embedding in paraffin in vacuo.

The embedding procedure *in vacuo* is preferred in order not to expose the dried specimens to the moisture of the atmosphere. The infiltration of paraffin is also facilitated. For this purpose a new technique for the heating of the tissue holders within the sample tube was developed. This was

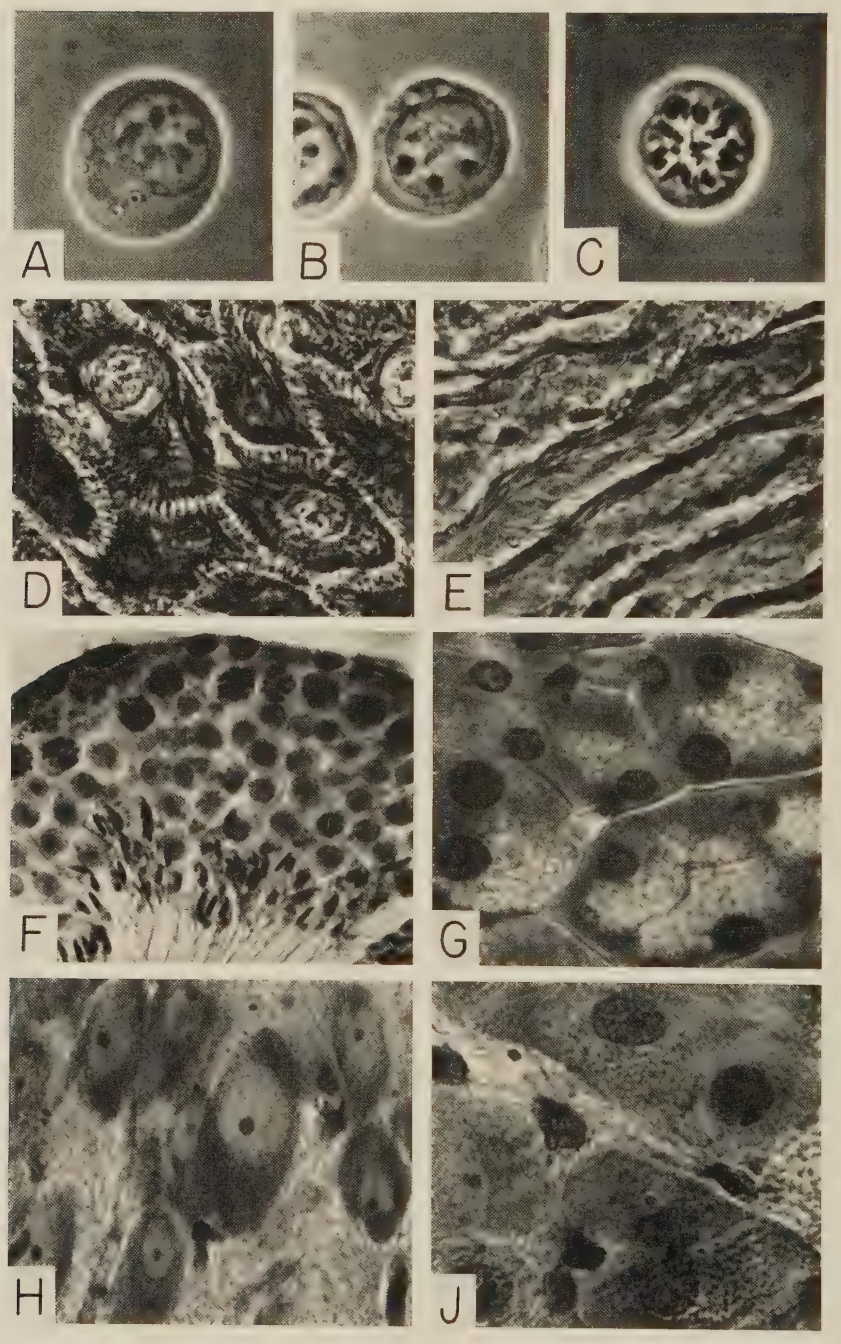
achieved by means of an oscillator. The wiring diagram of the oscillator circuit is shown in Fig. 6. The primary side of the transformer T_1 has six steps by which the secondary voltage can be varied in 100 volts steps between 500 and 1000 volts at a constant input of 220 volts A.C. Two self-excited oscillator triodes (RCA 1623) are used in Hartley coupling with a common oscillator circuit. The secondary voltage from the transformer (T_1) is used as anode voltage in the triodes, and therefore the tubes function alternately during the positive half periods. A high frequency A.C. of about 1.5 Mc is obtained in the common oscillator circuit (the inductance L_1 and its self-capacitance). Via the inductance (L_2) the high-frequency power is induced in the outer inductance (L_3), consisting of a removable copper wire loop outside the main oscillator (as shown in Fig. 5, J). The magnitude of the capacitance (C_3) is essential for obtaining maximal high-frequency power in the external loop (L_3). During the embedding procedure the loop is placed around the sample tube containing the tissue holders of iron. Heating of the tissue holders is caused by the energy of the electro-magnetic field in the centre of the loop (L_3).

The tissue holders were warmed to the melting point of the paraffin in 3–15 minutes, depending upon the secondary voltage chosen. When the paraffin started to melt at the edges of the individual grooves, the oscillator was put off in order to avoid overheating of the paraffin. The great mass of the metal holders secured complete melting of the paraffin blocks. The specimens were rapidly infiltrated with the melted paraffin and sank to the bottom of the grooves. After 10 minutes the paraffin was allowed to solidify. During the whole embedding procedure high vacuum was maintained.

SOME APPLICATIONS OF THE FREEZE-DRYING PROCEDURE ON DIFFERENT BIOLOGICAL MATERIALS

Fig. 7 serves to illustrate the results obtained when applying the freeze-drying procedure described in this paper.

Because of the minute size of the ice crystals in well preserved frozen-dried structures, phase-contrast or dark field illumination microscopy are the most reliable methods to estimate the morphological results of the freeze-drying procedure. *A*, *B*, and *C* show mouse ascites tumor cells (Krebs II) in the living state (*A*) and after freeze-drying (*B* and *C*). *D* and *E* illustrate certain structural elements in frozen-dried specimens after removal of the paraffin from the sections. The tonofibrils in the human epidermis are well preserved (*D*). In the aortic wall (*E*) the elastic fibrils can be seen to consist


A
B
C
D
E
F
G
H
J

of dense bundles of finer threads. *F* illustrates the preservation of the different cell layers in a tubule in the testis of the mouse. Tissues with a high lipid content are known to be difficult to freeze-dry. *G*, *H*, and *J* show a few examples of frozen-dried tissues with varying lipid contents.

SUMMARY

A freeze-drying apparatus built in metal with a minimum of glass parts and a new technique for the embedding in paraffin *in vacuo* is reported. The freeze-drying procedure with the apparatus is described and some applications on different biological material are given.

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Fig. 7 (opposite).
A. Mouse ascites tumor cell (Krebs II) in the living state. Phase contrast, × 870.
B. Mouse ascites tumor cell (Krebs II) after freeze-drying of a fresh squash preparation, in anhydrous glycerin. Phase contrast, × 870.
C. Same as *B*.
D. Human epidermis. Frozen-dried, 3 μ paraffin-section, deparaffinized in xylol Mounted in nonylic alcohol. Phase contrast, × 900.
E. Aorta from rat. Frozen-dried, mounted in nonylic alcohol. Phase contrast, × 900. (*E–J*, 3 μ paraffin-sections.)
F. Testis from mouse. Frozen-dried. Gallocyanin, × 500.
G. Pancreas from rat. Frozen-dried for 6 hours at —70° C. Gallocyanin, × 1 050.
H. Cerebral ganglion cells from rat. Frozen-dried for 8 hours at —70° C. Nissl-blue. × 710.
J. Medullar adrenal cells from rat. Frozen-dried for 12 hours at —70° C. Ehrlich hematoxylin-eosin. × 1 050.

BRIEF NOTES

INCORPORATION *IN VIVO* DE GLYCINE-1-^{14}C DANS LES CELLULES INDIVIDUELLES DE LA MOELLE OSSEUSE

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Reçu le 13 septembre, 1953

Dans un précédent travail (4), nous avons montré qu'il est possible d'étudier *in vitro* l'incorporation de glycine radioactive dans les cellules individuelles de la moelle osseuse, à l'aide d'une méthode autoradiographique permettant d'observer les traces produites, dans une émulsion photographique, par les particules β du ^{14}C.

Il nous a paru intéressant de suivre l'incorporation de cet acide aminé dans le même tissu, placé dans des conditions plus physiologiques que précédemment, c'est-à-dire *in vivo*.

On sait que le carbone du carboxyle de la glycine s'incorpore aux molécules protéiques ou aux groupes prosthétiques qui leur sont associés (acides nucléiques, cytochromes, hème) tant au cours d'expériences *in vivo* qu'*in vitro*. (1, 2, 5, 6.)

Conditions expérimentales. — Une solution physiologique contenant 5 μcuries de glycine-1-^{14}C est injectée dans la veine dorsale de la queue d'un rat. Après 20 heures, l'animal est sacrifié et la moelle osseuse est prélevée. Les cellules sont mises en suspension dans une solution de Ringer et elles sont ensuite lavées trois fois à l'aide du même milieu. Les frottis cellulaires, sur des lames microscopiques, préalablement gélatinées, sont fixés à l'alcool méthylique. Après étalement de l'émulsion (4), les lames sont exposées pendant 4 jours, comptés à partir du moment où l'émulsion est sèche. Le développement est effectué dans le révélateur « Amidol » (3) de composition suivante:

4,5 gr. sulfite de Na anhydre, 2 cc. KBr d'une solution à 10 %, et 4,125 g Diamidophénol (Johnson and Sons, London) dans 250 cc. d'eau distillée.

Ce révélateur présente sur le Kodak X, très alcalin (pH ~ 12) l'avantage d'avoir un pH proche de la neutralité, ce qui donne, dans le cas présent, de meilleurs résultats histologiques. Après fixation et lavage à l'eau, les lames sont plongées pendant 20 à 30 minutes dans de l'éthanol 70 %, ce qui favorise la diffusion des colorants. Les préparations sont enfin colorées pendant 30 minutes au Giemsa (Ciba) (5 gouttes de colorant par cc. de tampon au phosphate M/15, pH 7,4) et lavées à l'eau de ville. Ces quelques modifications de technique donnent de beaucoup meilleures colorations des cellules que celles obtenues précédemment.

Résultats expérimentaux. Les résultats de l'observation de près de 2000 cellules sont consignés dans le Tableau I. On constate, comme dans l'expérience faite *in vitro*, que la radioactivité des granulocytes diminue progressivement au cours de leur maturation; la concordance entre l'activité des cellules et leur degré de maturation est encore meilleure que dans nos expériences antérieures. Il est possible qu'*in vitro* les différentes catégories cellulaires ne se comportent pas toutes de manière identique, les unes continuant leur maturation à un rythme normal, les autres à une vitesse

TABLEAU I

	Nombre de cellules	Nombre de traces	Nombre de traces par cellule. $\times 10^2$
Hémocytoblastes	7	8	(114)[1]
Myéloblastes	27	20	74
Promyélocytes	107	51	52
Myélocytes neutro.	657	169	25
» eos.	26	12	46
Metamyélocytes neutro.	519	100	19
» eos.	56	25	44
Granulocytes neutro.	127	14	11
» eos.	32	6	(19)[1]
Proérythroblastes	3	3	(100)[1]
Erythroblastes basophiles	65	35	54
» polychromat.	34	9	26
» orthochrom.	1	0	—
Lymphocytes	83	13	16
Histiocytes	7	6	(86)
Plasmocytes	2	1	(50)
Plasmocytoblastes	1	2	(200)
Megacaryocytes	6	7	(116)[1]
Monocytes	2	1	(50)[1]
Total	1762	482	

ralentie. Remarquons en outre, que pour éviter l'infection bactérienne, l'incubation des cellules *in vitro* fut de plus courte durée (5 heures). Les cellules de la série rouge se comportent de manière identique *in vivo* et *in vitro*, de même d'ailleurs que les autres éléments du myélogramme.

Résumé et conclusions. Comme il avait déjà été observé *in vitro*, l'incorporation *in vivo* de glycine-1-^{14}C dans les cellules de la moelle osseuse est beaucoup plus considérable dans le cas des cellules jeunes, peu différenciées. Cette incorporation diminue au cours de la maturation des divers types de cellules tout comme dans les expériences de Morse (7) sur l'incorporation de phosphate marqué dans les éléments médullaires *in vivo*.

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[1] Les chiffres entre parenthèses résultant d'un nombre trop limité de cellule, n'ont qu'une valeur statistique approchée.

DISTRIBUTIONS OF CHYMOTRYPSINOGEN, PROCARBOXYPEPTIDASE, DESOXYRIBONUCLEASE, AND RIBONUCLEASE IN BOVINE PANCREAS[1]

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Received September 28, 1953

Antibodies labelled with fluorescein were introduced as specific histochemical reagents by Coons, Creech, Jones and Berliner (3). The method has been used most fruitfully for the localization of a number of foreign antigens in tissues (2). It was suggested that the same principle might be applied for the localization of individual proteins, including many enzymes and hormones, in the cells in which they are synthesized and stored (5).

The pancreas seemed a promising material for this type of study, both because of its intense activity in protein synthesis and because a number of its proteins were available in relatively pure form. This report describes the initial findings of an attempt to localize four such proteins, chymotrypsinogen, procarboxypeptidase, desoxyribonuclease, and ribonuclease, in the acinar cells of bovine pancreas.

Experimental. Antisera were prepared in rabbits against crystallized preparations of bovine chymotrypsinogen, carboxypeptidase, desoxyribonuclease, and ribonuclease. The techniques used in producing the antisera, in isolating and labelling the γ_2 globulin fractions, and in staining frozen-dried tissue sections were similar to those reported elsewhere (5). To prevent diffusion effects, sections were fixed by flotation on a buffered solution of formaldehyde in dioxane and water. This method will be reported in detail at a later date.

Thin sections (1–2 μ) of beef pancreas were prepared. Each was treated with one of the labelled antibody solutions. As controls, other sections were treated with labelled antibody solutions. As controls, other sections were treated with labelled globulin from normal rabbits. When the sections were examined in the fluorescence microscope, the staining pattern of each of the antibody solutions was sharply specific. Adjacent sections, in which individual acinar cells could be studied serially, revealed most accurately the differences in distribution of the four antigen preparations.

The results may be summarized as follows: Chymotrypsinogen was found only in the apex of the acinar cell and in the pancreatic juice within the acinar lumen and the duct system. The zymogen granules were intensely stained, as was the clear cytoplasmic matrix around the granules. The demarcation between the brilliantly

[1] Work done under a fellowship of the American Cancer Society recommended by the Committee on Growth of the National Research Council.

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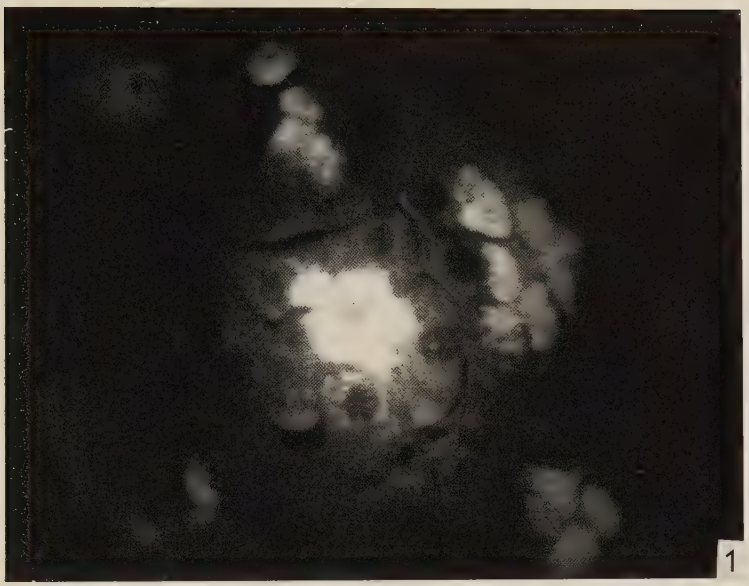

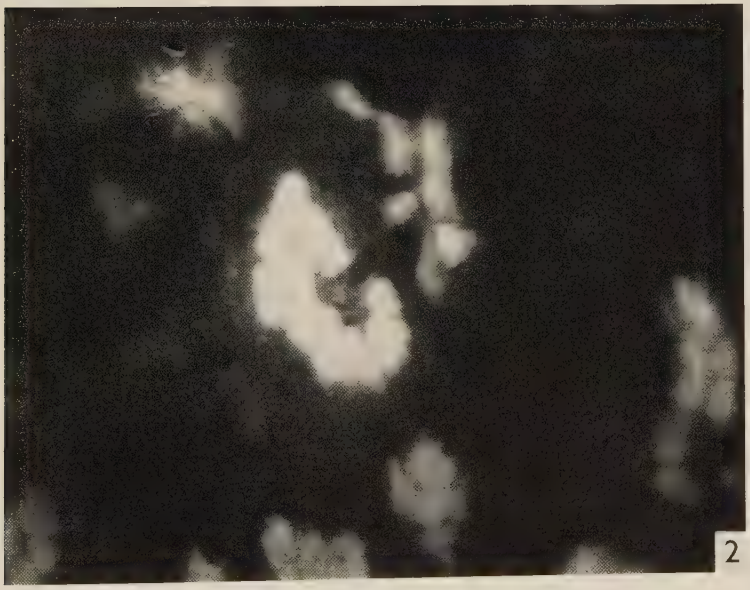

Figs. 1 and 2. Fluorescence photomicrographs of bovine pancreas. 2 μ sections. × 900.

Fig. 1. Stained with fluorescent antibody specific for carboxypeptidase. Zymogen granules and apical cytoplasm are strongly stained with yellow-green antibody. Mitochondria are visible by virtue of their blue autofluorescence, but are unstained.
Fig. 2. Stained with fluorescent antibody specific for chymotrypsinogen.

fluorescent apex and the remainder of the cell was abrupt; the nucleus, the mitochondria, and the basal cytoplasm were unstained.

Procarboxypeptidase (which reacted with antibody to carboxypeptidase) occurred in the same sites as chymotrypsinogen. In each acinar cell, it appeared that all the zymogen granules contained both.

The distributions of the depolymerases were more complex. Since recent reports have shown that each is a mixture of more than one protein (1, 4, 6), it is not yet possible to decide which component corresponds to each of the sites stained by antibody. However, the results indicate that each of the depolymerase preparations contained an antigen typically "zymogen" in distribution, present in the granules and apical cytoplasm of all acinar cells. In addition, each contained one or more antigens which were found throughout the cytoplasm of certain cells. Examination of adjacent sections revealed that ribonuclease and desoxyribonuclease antisera stained the cytoplasm of different sets of cells. Nuclei and mitochondria were not stained.

Discussion. The distributions of chymotrypsinogen and pro-carboxypeptidase could be determined to a resolution of less than 0.5 μ. The results indicate, that, at this level of organization, the acinar cells of the resting pancreas are all alike with respect to zymogen synthesis and storage; there is no specialization among the cells nor among the zymogen granules within each cell.

The findings for the depolymerases pose several problems for further investigation. Since the major component in each of the depolymerase preparations from pancreas is an enzyme apparently absent from other tissues (1, 4), it is suggested that this component may prove to be the antigen responsible for the "zymogen" distribution pattern in each instance. The minor components in both ribonuclease and desoxyribonuclease preparations are more likely to be the enzymes in pancreas which correspond to the depolymerases of other tissues (1). It is tentatively suggested that these may be the antigens distributed diffusely in the cytoplasm of certain acinar cells. No depolymerase antigens were found in mitochondria, although Schneider and Hogeboom reported that both ribonuclease and desoxyribonuclease were concentrated in mitochondria in homogenates of liver (7). It remains to be seen whether the homogenate technique and the antibody method will yield more consistent results if both are used to localize a single enzyme in the same tissue.

The author is deeply indebted to Dr. H. Holter and the staff of the Carlsberg Laboratory for advice and assistance, and to Dr. J. Orskov for the use of facilities at the State Serum Institute, Copenhagen.

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SHORT NOTE ON THE PRODUCTION OF NUCLEAR ARTEFACTS RESEMBLING MITOTIC FIGURES IN MICRO-ORGANISMS BY TREATMENT WITH ORGANIC SOLVENTS

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Received October 22, 1953

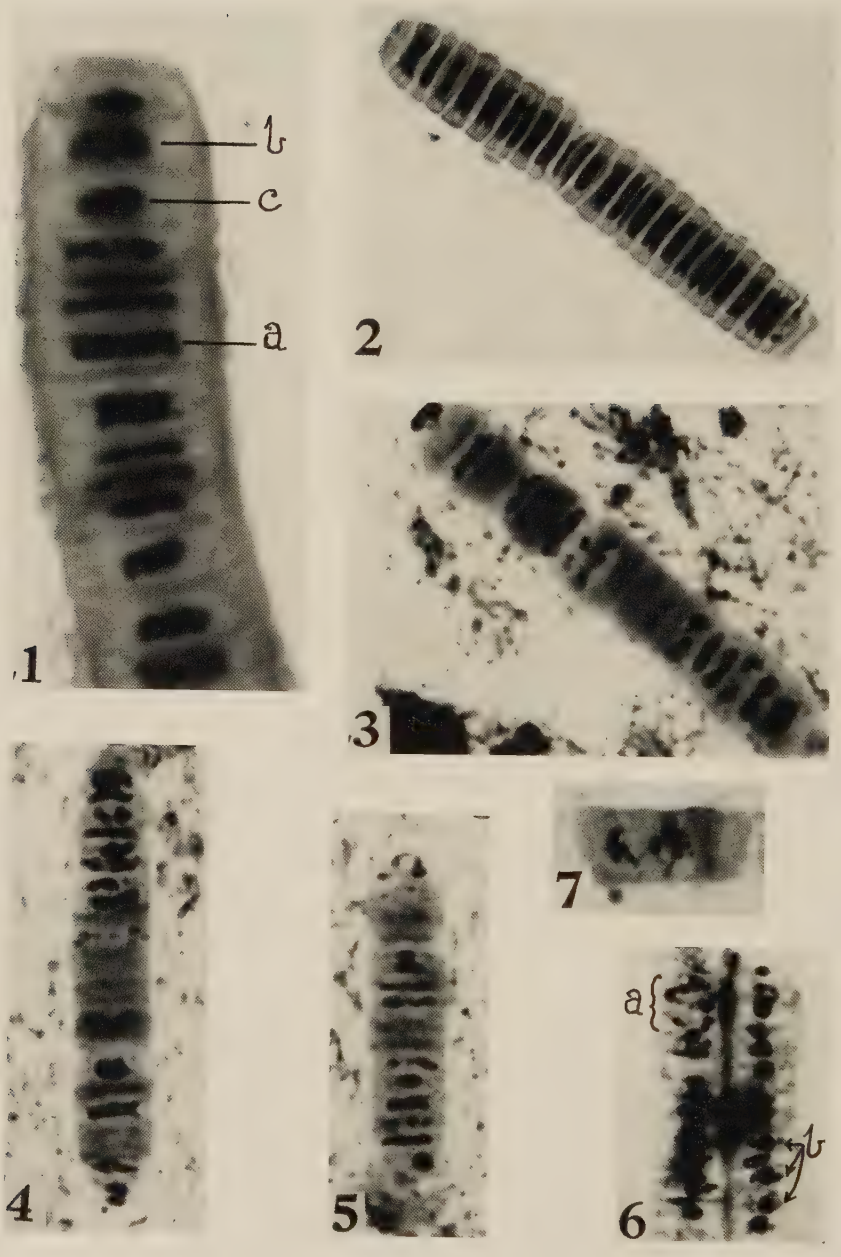
Numerous claims by DeLamater and Mudd (4, and other publications), to demonstrate mitotic spindles in bacteria, are open to a number of criticisms advanced by Bisset (1), that the method of dehydration entails transfer directly from water to concentrated solvents; that the complex structure of the septate bacteria examined has been ignored, and that DeLamater's illustrations are highly selective, showing very few organisms and including constant but unacknowledged republication of the same photomicrographs.

The structures described by DeLamater are so near the limit of microscopic resolution that any interpretation must of necessity be open to controversy. But the present note is intended to show how, in a micro-organism large enough to be clearly resolved, the effect of similar treatments is such as to bring about an almost complete destruction of the cellular organisation, with the production of obvious artefacts in the nuclear material, of such a nature that careful selection would permit the presentation of a series which might be capable of being mistaken for mitotic figures, in a less distinctly resolved preparation.

Similar observations have been made by Clark, Galyen and Webb (3) in smaller bacteria, and by Bisset (2).

The effect of absolute ethanol, acetone, ether and xylol upon cytologically stained bacteria, including *Bacillus megatherium* and *Micrococcus cryophilus* was examined. After examination in water-mounts, the preparations were dehydrated in these solvents at temperatures ranging from that of solid carbon dioxide, as recommended by DeLamater, to boiling point. The effect was always to cause gross distortion and damage to the cytological structures. Very occasional specimens resembled those illustrated by DeLamater. The temperature at which the dehydration was conducted made no difference whatsoever to the final result, but merely (as might have been anticipated) to the speed of the reaction. Robinow and Hannay (5) have confirmed the observation of Bisset (1) that even at low temperatures this method causes shrinkage, despite DeLamater's claims to the contrary.

The illustrations are of *Oscillospira* in guinea-pig caecal contents; this organism, which strongly resembles *Caryophanon* but is considerably larger, is typically bacterial in being composed of numerous cells, separated by cross-walls and containing paired rod-like nucleoids. Staining is by Robinow's modification of the Feulgen reaction (HCl-Giemsa). Figs. 1 and 2 are water-mounted, the remainder have been dehydrated in concentrated acetone for about 30 seconds, at room temperature, and mounted in cedar-wood oil.



Oscillospira, stained by acid-Giemsa. Fig. 1 × 2000, remainder × 1000. Description in text.

In Fig. 1 the paired nucleoids are shown in profile (*a*) and at various angles towards end-view (*b*, *c*). Fig. 2 illustrates the structure of the organism, consisting of about thirty disc-shaped cells, with a major division three-fifths of the way from one end. The same specimen, identified and rephotographed, in Fig. 3, shows the effect of acetone-dehydration. The debris is due to coagulation of the caecal contents. Further dehydrated specimens appear in Figs. 4 and 5, and Figs. 6 and 7 show excellent examples of false mitotic figures; *a* in figure 6 might be described as a "prophase, with centrioles appearing," and *b* as "condensed chromosomes between centrioles," whereas the right-hand nucleus in Fig. 7 simulates a "metaphase with one spindle and centriole visible," in the terms employed by DeLamater.

In this particular case, however, it is apparent that these appearances are meaningless artefacts, and that they are not even the most frequent types of artefact produced by this treatment; a situation which, in view of the exceedingly selective nature of his illustrations, may well be true also of De Lamater's material.

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A MODIFIED TECHNIQUE FOR ORGAN CULTURE *IN VITRO*

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Received October 26, 1953

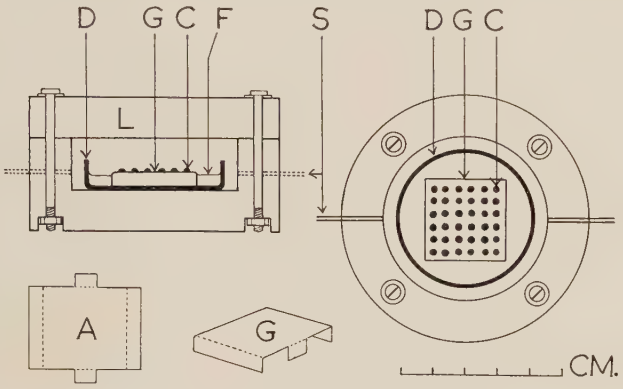
Techniques for the *in vitro* culture of small organs (1–2 mm^3 size) are now well established and have recently been reviewed (1). The main principle involved is that the organ is cultured on the surface of the nutrient medium, close to the gas phase from which it draws its necessary oxygen. The solubility of oxygen is so low and its diffusion so slow that an adequate supply to the centre of the culture is often a limiting factor, and if the culture is too large or if it is submerged in the medium, central necrosis occurs. This is particularly true of mature organs; embryonic ones can withstand a certain amount of anaerobiosis.

Most workers have cultured the organs on the surface of a solid medium such as plasma clot or agar jelly. I have previously described a method (2) in which organs (lymph nodes) were cultured on the surface of cotton-wool soaked in fluid culture medium. The method now to be described is a further development on the same lines; no new principle is involved, the novelty lies in the method used for supporting the cultures at the surface of a fluid medium.

A square piece of fine wire gauze has the edges bent over to form short legs and stands in a shallow glass dish. Sufficient fluid medium is added to reach and just wet the flat surface of the gauze grid. A piece of very thin soft tissue paper (lens paper) is now laid on the grid and the cultures are planted on the wet paper. The grid is made from tantalum wire gauze, which can be purchased from most surgical instrument houses ("Tantalum gauze, 0.003 inch diameter, 50 meshes per inch", Ethicon Suture Laboratories Inc., New Brunswick, N.J., U.S.A.). Tantalum is a non-toxic metal; it is an element not an alloy and therefore obtainable in very pure form. It is as strong as steel and is only attacked by hydrofluoric acid and strong alkalies. The grids are cleaned initially, and after each use, in boiling 2 per cent sodium oleate, then in acetone and finally in pure nitric acid overnight. The paper used is Green's Lens Tissue No. A 105, cleaned by soaking in Ether A.R., 4 changes, followed by glass distilled water, 12 changes over 24 hours, after which it is dried and cut to size. The dry papers and grids are sterilised at 120° C.

The apparatus can be made to almost any dimensions. The one we use, which carries up to 36 cultures in 5 ml of medium, is shown in Fig. 1 and is constructed as follows. A piece of tantalum gauze is cut out to the shape A and then folded along the dotted lines to give shape G. The cut wire ends along the edges are bent over to prevent fraying of the gauze. The surface of the grid (G) is 25 × 25 mm and the legs are 4 mm high. The glass dish (D), 4 cm in diameter and 1 cm deep, is made by cutting down a 25 ml crystallizing dish. After adding enough medium to cover the grid, the lens paper is carefully laid on. Medium is now removed until the outer fluid level

(F) is about 1 mm lower than the paper; but this level is not critical, it can vary somewhat without altering the wetness of the paper. The cultures (C) are planted on the paper and the dish is then placed in a thick-walled Perspex (Lucite) chamber of 30 ml capacity. A piece of wet filter paper on the floor of the chamber prevents the dish from sliding about. This filter paper is wet with 1 per cent NaCl, or, if is is desired to absorb the respiratory CO_2, 1 per cent NaOH. These solutions have about



the same vapour pressure as the culture medium, so water does not distil to or from the medium. The lid (L) is bolted on and the chamber placed in an incubator or a water bath at 37° C. If the perspex surfaces have been truly faced in the lathe it is not necessary to grease the joint. Two stainless steel tubes (S), tightly fitted through the walls of the chamber, have rubber tubes attached so that any desired gas mixture can be passed through the chamber. After the chamber has warmed up we usually fill it with oxygen by passing 400 ml through. The oxygen is first bubbled through a water column and then passed through a sterile cotton-wool filter, the whole apparatus being immersed in the 37° C water bath, so that the gas is warm, water-saturated and sterile before it reaches the culture chamber. With 20 or more lymph-node cultures in the chamber it is necessary to refill with oxygen every day and to change the medium every two days.

This method was primarily designed for studying the nutrition and metabolism of organs cultured *in vitro*, for which purpose it has the following advantages.

1. A large number of cultures is used in each vessel, so that metabolic changes are considerable and adequate material is available for analysis.

2. Samples of medium can be removed for analysis, and additional nutrients or drugs can be added, at any stage of the experiment.

3. The medium can be changed and the cultures can be washed without disturbing their position.

4. Inert supporting material (grid and paper), which may retain or adsorb constituents of the medium, is reduced to a minimum.

5. Synthetic fluid media of exactly known chemical composition can be used. The gaseous environment is also under complete control.

We have used four of these culture chambers for 5 months (1500 cultures), in a study of the survival and metabolism of rat lymph nodes in synthetic media, and have found the method simple and reliable. In the best synthetic medium so far devised the lymph nodes contained about 2 per cent of dead cells after 4 days *in vitro*.

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Experimental Cell Research, **6**, *249–250 (1954)*

LIBERATION OF PROTEOLYTIC ENZYMES OF THE SEA URCHIN EGG BY RIBONUCLEASE

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Received November 11, 1953

Sea urchin eggs contain several proteolytic enzymes (2, 3, 4) having various pH optima. Two of these with pH optima at 6.7 and 7.7 are of special physiological interest, as they probably are of importance in fertilization. On several occasions it has been noticed that these enzymes have a high activity in extracts of some unfertilized egg batches whereas they have a low or sometimes indetectible activity in extracts of other unfertilized eggs. However, in fertilized eggs, the activity of these enzymes is remarkably more constant. The varying activity of the enzymes in unfertilized eggs and the regular appearance of these enzymes in fertilized eggs indicate that the enzymes may be more or less completely bound (or inhibited) in unfertilized eggs but that they can be set free either before fertilization by unknown factors or at fertilization by a normal process. As a matter of fact, it has been shown (2, 3) that the enzymes are somehow related to granules in the cytoplasm. Assuming that these enzymes actually occur in the unfertilized eggs but are blocked by or bound to some substance, it seems plausible that they are liberated at fertilization by an enzymatic process and that it might be possible to liberate them *in vitro* in an homogenate of unfertilized eggs, if the proper enzyme were added. A similar problem has been investigated by Myrbäck (5, 6). He found that β-amylase of barley is partly bound to insoluble proteins and is liberated at germination; the bound enzyme can also be liberated at extraction of ungerminated barley if a proteolytic enzyme such as papain is added.

In the present case the question arose whether the proteolytic enzymes might be bound to nucleic acids and could be liberated by ribonuclease.

Experimental. *Paracentrotus lividus* eggs, which had been frozen-dried in 1952, were used in the first experiment. 0.25 g of that was suspended in a mixture of 8 ml of 1 M KCl and 2 ml of 0.03 per cent crystalline ribonuclease (Worthington) solution. For control 0.25 g was suspended in a mixture of 8 ml of 1 M KCl and 2 ml of water. The suspensions were frozen, thawed and kept in a refrigerator for 12 hours, whereupon they were centrifuged. The proteolytic activity of the supernatant was measured by its effect on the viscosity of gelatin (Ostwald viscosimeter) at pH 6.7 and 7.6 in the presence of cysteine; the results are given in Table I.

Similar results were obtained also with eggs of other sea urchin species. Suitable controls showed that the presence of ribonuclease in the assay mixture did not influence the determination of proteolytic activity.

Conclusion. Two proteolytic enzymes obtained from extracts of sea urchin eggs and having pH optima at pH 6.7 and 7.7 respectively can be liberated by ribo-

nuclease. As ribonuclease is present in eggs (1) and in spermatozoa it is most likely that this reaction in vitro corresponds to the same reaction *in vivo*. This is a confirmatory example of the fact, emphasized by Runnström, that the initiation of the development of the egg after fertilization is due to an activation of enzyme systems (7).

TABLE I

Proteolytic activity (mμA/ml (1) after extraction in presence and absence of added ribonuclease.

pH	Ribonuclease	Water
6.7	50	0.7
7.6	10	0

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Experimental Cell Research, **6**, *251–253 (1954)*

THE LOCALIZATION OF RADIOSULFATE IN BONE TISSUE[1]

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Received November 28, 1953

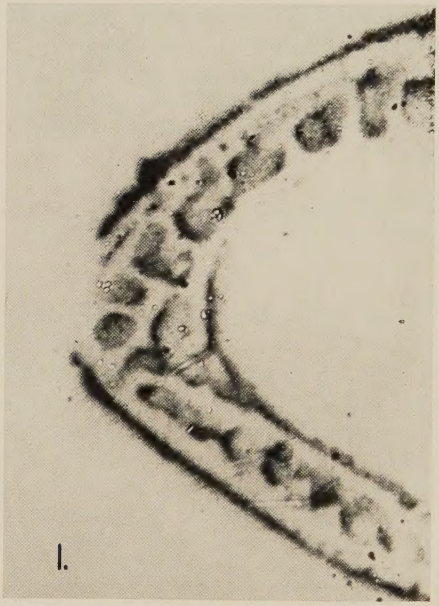
Recent studies on the uptake of radioactive sulfate in the mammalian organism have shown that several tissues take up considerable amounts of labelled sulfate shortly after the injection of a single dose $Na_2S^{35}O_4$. It is particularly ester sulfuric acid containing tissues such as cartilage, skin, aortic wall and bone marrow that have a rapid uptake of radiosulfate (3, 6, 2). Dziewiatkowski (4) in his autoradiographic studies on the articular cartilage and bone of suckling rats, observed a faint uptake of S^{35} just beneath the heavily labelled epiphyseal cartilage, indicating uptake of radiosulfate in the bone tissue. The present communication is a preliminary report of the results obtained when applying autoradiographic, microradiographic and chemical procedures to the study of the localization of radiosulfate in bone tissue.

In these experiments, two normal littermate dogs, three and a half months of age and weighing about 7 kg, were used. One dog received 2 mC carrier-free $Ca^{45}Cl_2$ intravenously and served as a control and the other received 10 mC carrier-free $Na_2S^{35}O_4$. Both dogs were sacrificed 4 days after the injections and their bones were freed from soft tissues and fixed in absolute alcohol. Thin longitudinal and cross sections were cut with a rotating saw. These sections were ground on glass plates to a thickness of 50 to 100 μ. Autoradiograms were recorded on Agfa Printon film and the exposure time amounted to 14 days. The distribution of mineral salts and the degree of mineralization in the same sections was studied by microradiography (1).

Both longitudinal and cross sections from the long bones of the dog which had received sodium sulfate showed considerable uptake of isotope in the bone tissue itself. In the longitudinal sections, the articular cartilage and the epiphyseal plate showed an extraordinarily high uptake of sodium sulfate as could be expected. Immediately below the heavily labelled epiphyseal plate there was a broader zone, which showed an appreciable uptake of sulfate. Further down in the diaphysis there was a considerable periosteal and endosteal uptake and in the compacta, places were found which had a relatively good labelling.

The cross sections showed a rather high uptake in the periosteal and endosteal layers. Certain Haversian systems and the lining of some resorption cavities showed considerable labelling. The microradiograms of the cross sections showed the differences in mineralization which are characteristic for the rebuilding processes of the bone tissue. The dogs used in our experiments were still growing, and thus

[1] This paper is part VII of a series "Biophysical Studies on Bone Tissue".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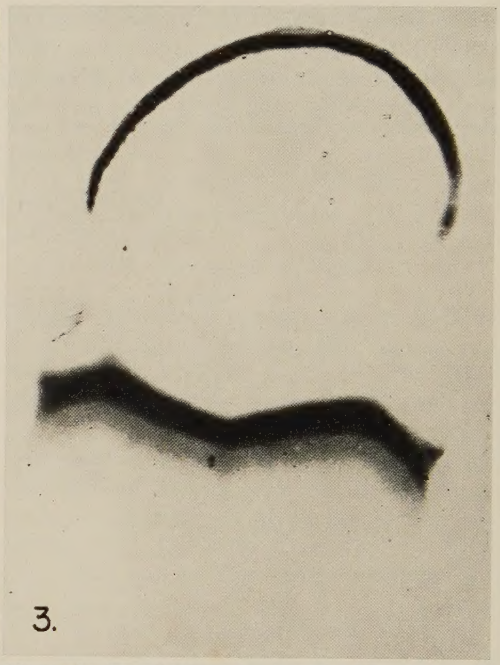
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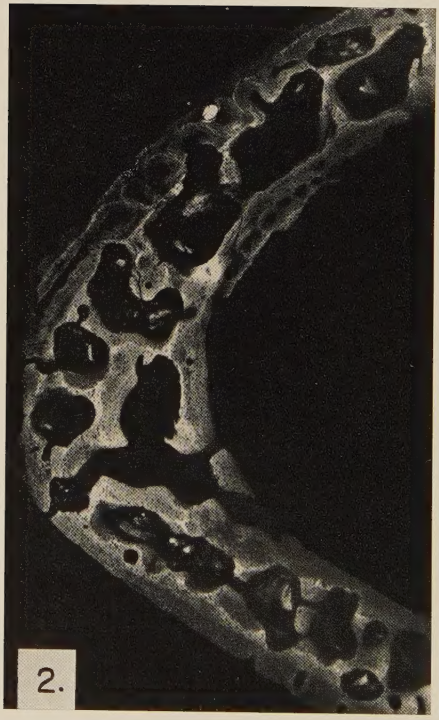
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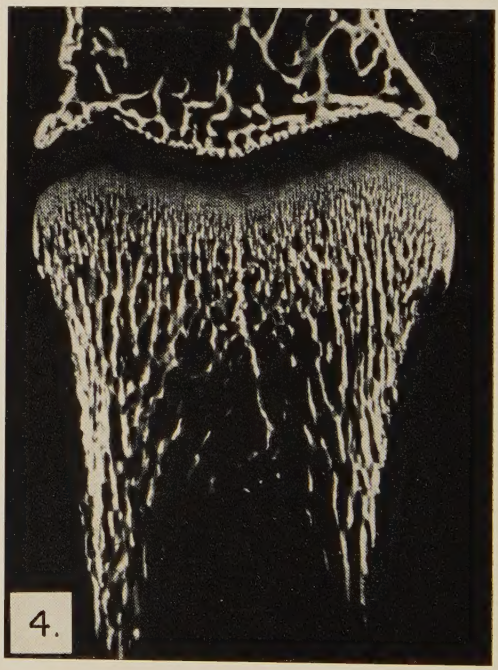
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bone rebuilding was going on at a rapid rate. Therefore the compacta showed a great number of resorption cavities often partly lined by newly formed, less mineralized bone tissue.

It has been shown by us that after administration of P^{32} (5) or Ca^{45} the main uptake in the bone tissue is found in those structures which have a low content of mineral salts, that is, in newly formed bone tissue. When the autoradiograms obtained from the bone tissue of the dog labelled with Ca^{45} were compared with those from the S^{35} dog it was evident that identical structures were labelled in both instances. Calcium and phosphorus are found in high concentrations in the bone tissue. The calcium is localized almost exclusively and phosphorus to a large extent in the inorganic (apatite) fraction of the bone tissue. Sulfate, however, is not recognized as a normal constituent of the bone salt (apatite).

The rebuilding processes take place at a higher rate in the spongious than in the compact bone. Powdered spongiosa from the sulfate labelled dog showed about twice as high radioactivity as powdered compacta. About half of the radioactivity (identified as inorganic sulfate) of the bone powder could be extracted with water. It seems likely that at least part of the radioactive sulfate was localized in the inorganic part of the bone tissue. This is supported by the fact that bone slices from the "sulfate dog" refluxed with ethylene-diamine, which removes the organic constituents, still gave distinct radioautographs. As decalcified sections of bone also gave good autoradiographs, part of the activity must be bound to the organic fraction.

The results of *in vitro* labelling of bone tissue with $Na_2S^{35}O_4$ will be published shortly.

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Fig. 1. Radioautograph of a cross section of the femur from a dog given $Na_2S^{35}O_4$. Localized uptake of the isotope.
Fig. 2. Microradiogram of the section the radioautograph of which is shown in Fig. 1. Areas which are light in the photograph have a high mineralization. Note the spaces for the bone cells.
Fig. 3. Radioautograph of a longitudinal bone section. High uptake of S^{35} in the cartilage but also some in the bone tissue beneath the cartilage.
Fig. 4. Microradiogram of the section in Fig. 3.

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